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ABSTRACTS OF PAPERS PRESENTED AT THE FORTY-THIRD ANNUAL MEETING OF THE SOCIETY OF GENERAL PHYSIOLOGISTS

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Marine Biological Laboratory

Woods Hole, Massachusetts

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CONTRIBUTED PAPERS

1. A Pertussis-sensitive G Protein Mediates Both Activation of "S"-like K^+ Current and Suppression of Ca^{2+} Current by a Family of Neurotransmitter Receptors in *Aplysia* Neurons V. BŘEZINA,* S. S. VOGEL,* G. J. CHIN,* and J. H. SCHWARTZ,* *Howard Hughes Medical Institute, Columbia University, New York* (Sponsor: D. L. Armstrong)

Aplysia neurons have been proposed (Březina. 1988. *J. Physiol.* 407:15; Březina. 1988. *Soc. Neurosci. Abstr.* 14:754) to possess a family of "slow" neurotransmitter responses consisting of activation of a K^+ current resembling the "S" current and simultaneous suppression of the Ca^{2+} current, both mediated by a single distinct receptor for each transmitter. We have combined electrophysiological and biochemical techniques to study the involvement of G protein(s) in these responses, concentrating on those in neurons L10 (to acetylcholine, histamine, and the neuropeptide FMRFamide) and R2, LP1, and the LUQ cells L2-L6 (to acetylcholine and FMRFamide only). Injection into the voltage-clamped cells of GTP-γ-S mimicked the transmitter responses, slowly but irreversibly increasing "S"-like K^+ current and suppressing Ca^{2+} current. Full development of these effects of GTP-γ-S blocked the ability of each of the transmitters to modulate these currents further, but did not, in the same cells, affect an acetylcholine-induced Cl^- current and a FMRFamide-induced Na^+ current thought to be mediated by separate "fast" receptors. Another probe of G-protein function, pertussis toxin (PTX), has been found to inhibit transmitter-activated slow K^+ currents in other *Aplysia* neurons (Sasaki and Sato. 1987. *Nature.* 325:259; Sasaki et al. 1987. *Jap. J. Physiol.* 37:551; Volterra and Siegelbaum. 1988. *Proc. Natl. Acad. Sci.* 85:7810), and also, under current clamp, the histamine-induced hyperpolarization in L10 (Vogel et al. 1987. *Soc. Neurosci. Abstr.* 13:597). We found that injection into voltage-clamped cells of PTX slowly (>90% within 2 h) blocked both the activation of "S"-like K^+ current and the suppression of Ca^{2+} current due to each transmitter. Biochemical characterization of G proteins using PTX-catalyzed [^{32}P]ADP-ribosylation and Western blotting revealed only one PTX substrate in *Aplysia* neuronal membranes, a M_r 40,000 component recognized by an antiserum directed against the α subunit of bovine-brain G_o (Vogel et al. 1989. *Brain Res.* 478:281). Large quantities of this G protein were found in single isolated R2 (56 ± 5 fmol/cell, $n = 6$), L10 (20 ± 3 fmol/cell, $n = 3$), and LUQ cells. Since no other PTX substrate was found, this G protein presumably mediates all PTX-sensitive processes in these cells, including the "slow" neurotransmitter responses.

2. Norepinephrine and GTPγS Inhibit a Calcium Conductance and Activate a Nonselective Cation Conductance in Rat Parasympathetic Cardiac Neurons D. J. ADAMS and Z. XU,* *Department of Pharmacology, University of Miami School of Medicine, Miami, Florida*

The voltage-dependent and kinetic properties of a calcium conductance and its modulation by norepinephrine (NE) were investigated in cultured parasympathetic neurons from rat intracardiac ganglia. Whole-cell calcium currents were recorded with an intracellular patch pipette solution containing: 100 mM CsCl, 2 mM MgATP, 10 mM Cs_2BAPTA, 40 mM HEPES-CsOH, pH 7.2, and in the presence of 300 nM tetrodotoxin and 50 mM tetraethylammonium ion extracellularly. Inward current amplitude and rate of inactivation were dependent on the $[Ca^{2+}]_o$. The current-concentration relationship for Ca^{2+} saturated (K_d 4 mM), and Ba^{2+} and Na^+ (in the absence of $[Ca^{2+}]_o$) were permeant. The calcium currents evoked from a holding potential of −100 mV were predominantly high-threshold and exhibited <20% inactivation (in 5 mM Ca^{2+}) during a 500-ms depolarizing pulse to +10 mV. This calcium current was completely blocked by ω-conotoxin GVIA (30 nM), amlodi-

Asterisks indicate authors who are not members of the Society of General Physiologists.

pine maleate (30 μM), and cadmium ion (30 μM), but not amiloride (30 μM). NE (<100 μM) reversibly inhibited the current carried by either Ca^{2+} or Na^+ through calcium channels and this effect was antagonized by the α-antagonist, phentolamine. NE evoked the simultaneous activation of a voltage-sensitive outward current which was rapidly activated by depolarization and did not exhibit time-dependent inactivation. This outward current could be carried by internal Cs^+ or choline$^+$ but not L-arginine$^+$. The replacement of external Cl^- with SO_4^{2-} did not affect this current. The inhibition of inward calcium current and activation of outward current by NE were mimicked by the addition of 100 μM GTPγS but not by 100 μM GDPβS to the pipette solution. These data suggest that these α-adrenergic effects in parasympathetic cardiac neurons are mediated via a G protein. [Supported by NIH grant HL-35422.]

3. ADP-Ribosylation and Immunological Characterization of GTP-binding Proteins That Mediate Presynaptic Inhibition GEORGE G. HOLZ,* TIMOTHY J. TURNER,* and KATHLEEN DUNLAP, *Department of Physiology, Tufts University School of Medicine, Boston, Massachusetts*

Regulation of voltage-dependent calcium channels by GTP-binding proteins (G proteins) is a likely means by which inhibitory transmitters suppress excitation-secretion coupling in presynaptic nerve endings. We previously reported that in chick dorsal root ganglion neurons presynaptic inhibition is blocked by prior exposure of cultures to pertussis toxin (PTX). Here we report that in these same cells, PTX catalyzes ADP-ribosylation of G proteins migrating on SDS-PAGE (1-D gel electrophoresis) as a doublet of M_r 40–41 kD. Isoelectric focusing combined with SDS-PAGE (2-D gel electrophoresis) demonstrates that this doublet is resolvable as four distinct spots with pI of ~5–6. Western immunoblot analysis of 1-D gels using antisera directed against synthetic peptides corresponding to amino acid sequences predicted from cDNAs for PTX-sensitive G protein α subunits reveals a minimum of two G_i-like proteins (M_r 40 and 41 kD) and a third G_o-like protein (40 kD). Subcellular fractionation of chick cerebral cortical homogenates by sucrose density gradient centrifugation indicates that these ribosylatable, immunoreactive G proteins are included in the synaptosomal plasma membrane and synaptic vesicle fractions. We conclude that G_i and G_o may regulate transmitter release not only at the level of calcium entry, but also at subsequent steps in the secretory pathway.

4. Muscarinic Modulation of Ca Current in Bullfrog Intracardiac Parasympathetic Neurons Involves Pertussis Toxin–sensitive GTP-binding Proteins AMY TSE,* ROBERT B. CLARK,* and WAYNE GILES, *Departments of Physiology and Medicine, University of Calgary, Calgary, Alberta, Canada*

Acetylcholine is known to inhibit its own release from intracardiac parasympathetic neurons during vagal stimulation. To examine the mechanism involved in this negative feedback regulation, we investigated the effect of ACh on the Ca current of isolated intracardiac parasympathetic neurons from bullfrog (*Rana catesbeiana*) using the whole-cell patch-clamp method. ACh reversibly reduces the Ca current ($K_d \simeq 20$ nM). This response is voltage dependent with the maximum inhibition of peak Ca current (66.4 ± 2.4%, $n = 6$) occurring near the peak of the current-voltage relation (≃ +15 mV). The ACh response can be mimicked by carbachol, oxotremorine, pilocarpine, *dl*-muscarine, and antagonized by atropine, suggesting that this modulation is mediated via muscarinic receptors. Three lines of evidence support the involvement of pertussis toxin–sensitive GTP-binding proteins: (*a*) incubation of neurons with 200 ng/ml of pertussis toxin for 12–24 h almost completely abolishes the muscarinic response ($n = 8$) while incubation with heat-inactivated pertussis toxin fails to alter it ($n = 4$): (*b*) inclusion of the nonhydrolyzable GTP analogue GTPγS (50–200 μM), in the pipette mimicks the muscarinic effect; (*c*) inclusion of the nonhydrolyzable analogue of GDP, GDPβS (2–5 mM), almost completely inhibits the response. Application of dibutyryl cAMP, 8-bromo-cGMP, phorbol esters, and diacylglycerol analogues does not mimic or inhibit this muscarinic effect, suggesting that adenylate cyclase, guanylate cyclase, and protein kinase C pathways were

unlikely to be involved. One possible mechanism for this modulation may be a direct coupling of GTP-binding protein(s) with the Ca channels. [Supported by the Canadian Medical Research Council, the Canadian Heart Foundation, and the Alberta Heritage Foundation for Medical Research.]

5. Regulation of Spontaneous Opening of the Muscarinic K^+ Channel in Rabbit Atrium M. KAIBARA,* T. NAKAJIMA,* H. IRISAWA,* and W. GILES, *Department of Medical Physiology and Medicine, University of Calgary, Calgary, Alberta, Canada*

One type of K^+ channel in rabbit atrial cells is activated by muscarinic agonists and depends upon G proteins. Cell-attached patch recordings show low frequency channel activity of this type even in the absence of agonists. We have investigated the controlling mechanism for these spontaneous events by applying the excised patch-clamp technique to isolated single myocytes from rabbit atrium. The channel activity disappeared immediately after patch excision, but it reappeared after application of ATP (1 mM) and $MgCl_2$ to the internal side of the membrane. The channel activity (NP_o) was dependent upon the concentration of free Mg^{2+}; it was half-maximal at 2.2×10^{-4} M. After the channel had been activated by 100 μM guanosine 5′-*o*-(3-thiotriphosphate) (GTPγS) together with ATP and Mg^{2+}, a further increase in the Mg^{2+} concentration failed to enhance the response. Thymidine 5′-triphosphate (TTP) (1 mM) and guanosine 5′-triphosphate (GTP) (1 mM) also activated this K^+ channel in the presence of 2 mM $MgCl_2$; however TTP and GTP were much less effective than ATP. Both guanosine 5′-*o*-(2-thiodiphosphate) (GDPβS) and *N*-ethylmaleimide (NEM) inhibited the ATP-induced opening of this channel. In contrast, the channel activity induced by GTPγS was not abolished by NEM or GDPβS. The channel activity induced by ATP was decreased by simultaneous application of guanosine 5′-diphosphate (GDP) (25 μM). In combination, these data indicate that the spontaneous opening of the muscarinic K^+ channel is mediated by G proteins and that activity of G proteins is regulated by nucleotides, and Mg^{2+} in the absence of agonists. [This work was supported by the Canadian Medical Research Council and Heart Foundation, and the Alberta Heritage Foundation for Medical Research.]

6 GTP-binding Protein Regulates the Activity of Na^+ Channels from the Epithelial Cell Line A6 HORACIO F. CANTIELLO,* CHARLES R. PATENAUDE,* and DENNIS A. AUSIELLO, *Renal Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts*

The ability of GTP-binding proteins to regulate a Na^+-conducting channel in the apical membrane of the toad kidney cell line, A6, was examined by the patch-clamp technique. A Na^+ channel was observed that did not display any detectable rectifying properties between -100 and $+100$ mV, was selective for Na^+:K, 5:1, and had a conductance of 8.87 ± 0.34 pS (78) in Na^+ symmetrical solutions (Na^+ = 115 mM). Single-channel activity was amiloride-sensitive in a dose-dependent manner with an apparent $K_i < 10^{-7}$ M. These data are in close agreement with those previously reported by Hamilton and Eaton (1985. *Am. J. Physiol.* 249:C200–C207). The regulatory role of GTP-binding proteins on Na^+ channel activity was examined by recording inside-out single-channel activity with the patch clamp. Single-channel activity, expressed as the percentage of total open time, was reduced by 85.2% after 1 min perfusion with pertussis toxin (PTX) and NAD (46.5 ± 9.29 [7] vs. 6.87 ± 2.49 [5], $P < 0.001$) and declined to zero in ~3 min. The single-channel conductance remained constant at 9.35 ± 2.35 (7) vs. 8.84 ± 2.19 (5) pS. In contrast, GTP (10^{-4} M) increased spontaneous channel activity from 6 to 50% of open time. GDPβS decreased spontaneous channel activity by 84.1% (43.8 ± 13.6 [3] vs. 6.95 ± 4.38 [5], $P < 0.025$) also without effect on the ionic conductance. The GTPγS-activated, purified α subunit of the human G_i protein, α_i-3 (20 pM), reversed the effect of PTX, 32.4 ± 13.1 (3) % open time as compared with the control in the presence of PTX, 0%, $P < 0.05$. These data indicate that amiloride-sensitive epithelial Na^+ channels are regulated by a G_i-like, GTP-binding protein, whose activity is replaced by the α_i-3 subunit.

7. Cyclic AMP and IP_3 Link G Protein-coupled Chemoreceptors to Ion Channels in Olfactory Cilia RICHARD C. BRUCH,* DIEGO RESTREPO, and JOHN H. TEETER,* *Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois; and Monell Chemical Senses Center, Philadelphia, Pennsylvania*

High affinity amino acid chemoreceptors in the olfactory system of the channel catfish (*Ictalurus punctatus*) are coupled to one or more heterotrimeric G proteins (Bruch and Kalinoski. 1987. *J. Biol. Chem.* 262:2401–2404). We have therefore investigated the ability of stimulus amino acids from each of the four receptor subtypes (Caprio and Byrd. 1984. *J. Gen. Physiol.* 84:403–422; Bruch and Rulli. 1988. *Comp. Biochem. Physiol.* 91B:535–540) to support quanine nucleotide–dependent cAMP and IP_3 formation in isolated cilia. Ten stimuli elicited similar increases in cAMP formation, although significant activation of adenylate cyclase was observed only when receptor occupancy approached or exceeded 50%. Cyclic AMP reversibly increased membrane conductance by gating nonselective cation channels in ciliary membranes incorporated in phospholipid bilayers. Stimulus amino acids also evoked rapid and transient increases in IP_3 formation in isolated cilia. Consistent with this result, stimuli also elicited rapid and transient increases in intracellular calcium in dissociated fura 2-loaded olfactory neurons. This response was abolished by removal of extracellular calcium, which suggests that the increase in Ca_i was mediated by calcium influx. In addition to releasing calcium from isolated olfactory microsomes, IP_3 also gated a calcium channel in ciliary membranes incorporated in phospholipid bilayers. Taken together, these results suggest that at least two G protein-linked second messengers mediate olfactory transduction by modulating ion channels underlying membrane depolarization. [Supported by NIH grant NS-26927 and NSF grant BNS8996179.]

8. Novel K^+ Channels in Cardiac Cells Activated by Arachidonic Acid and Phospholipids DONGHEE KIM and DAVID E. CLAPHAM, *Department of Physiology and Biophysics, Chicago Medical School, North Chicago, Illinois; and Department of Pharmacology, Mayo Foundation, Rochester, Minnesota*

We have shown recently that arachidonic acid (AA) activated a muscarinic-gated K^+ channel in cultured rat atrial cells (Kim et al. 1989. *Nature.* 337:557). During our experiments with AA, we noted the appearance of another type of K^+-selective current in rat atria ($I_{K,AA}$). In whole-cell patch clamp, AA (10 μM) in the pipette gradually increased slope conductance from 0.39 to 1.12 nS at 40 mV, and from 0.46 to 1.00 nS at −120 mV, (4 mM $[K]_o$, 140 mM $[K]_i$). The inward K^+ current was blocked by 1 mM extracellular $BaCl_2$ and the outward K^+ current was blocked by replacing intracellular KCl with CsCl. Studies using inside-out patches showed that in symmetrical 140 mM $[K^+]$, AA (10 μM) activated a K^+-selective channel with conductances of 160 (outward) and 70 pS (inward) with a mean open time of ~1 ms at 50 mV. A plot of the reversal potential vs. $[K]_i$ had a slope of −56 mV/decade, close to the expected Nernst slope of −58 mV/decade at 22°C. The channel activity was not affected by ATP, Mg^{++}, Ca^{++}, GTPγS, GTP, or CHAPS. Interestingly, the channel activity increased 4- and 10-fold by decreasing the bath pH to 6.8 and 6.4 from 7.2, respectively, indicating that H^+ modulates the K^+ permeability of this channel. Other fatty acids (linoleic, but not oleic, palmitic, or stearic acids) activated the same K^+ channel. Phosphatidylcholine and phosphatidylserine, from which free fatty acids are generated by the action of phospholipase A_2, activated a second K^+-selective channel $I_{(K,PL)}$ when applied to the intracellular surface of the patch. This channel had a lower conductance (68 vs. 160 pS) compared to $I_{K,AA}$. Metabolites of AA such as HETE, HPETE, and leukotrienes did not cause channel activation. The K^+ channels found in this study may be important in ischemic conditions where pH_i is lowered and intracellular levels of certain free fatty acids are elevated. The K^+ channels may also be part of new second messenger pathways involving lipophilic compounds. [Supported by NIH grants HL-34873 and HL-40586.]

9. GTP-binding Proteins Mediate Dopamine Activation of a Potassium Current in Identified Rat Lactotrophs L. C. EINHORN* and G. S. OXFORD, *Curriculum of Neurobiology, University of North Carolina, Chapel Hill, North Carolina*

Dopamine (DA) is the major physiological regulator of prolactin (PRL) secretion from the anterior pituitary, exerting a tonic inhibitory control. Utilizing the reverse hemolytic plaque assay and whole-cell patch-clamp techniques, we investigated the actions of DA on the membrane potential and ionic conductances in primary rat lactotrophs as well as the signal-transducing mechanisms which may be involved. Application of DA evoked a hyperpolarization that was D2 receptor-mediated and associated with an increased K^+ conductance. Whole-cell current responses to ramp voltage commands revealed that the DA-activated K^+ current exhibited a slight inward rectification and was present when both the extracellular and intracellular Ca^{++} concentrations were buffered to a nominal level or when cells were dialyzed with 2 mM cAMP. The current was insensitive to TEA (10 mM) and only partially blocked by 4AP (5 mM). Extracellular quinine (100 μM) completely inhibited the inward current while only partially inhibiting the outward current. Pretreatment of cells with pertussis toxin (PTX) or intracellular dialysis with GDPβs (100–500 μM) abolished the DA responses. In the absence of DA, intracellular GTPγs dialysis (50–100 μM) produced a slowly developing membrane hyperpolarization which was comparable to that produced by DA in both the amplitude and associated increase in conductance. Furthermore, early application of DA in a GTPγs-dialyzed cell resulted in a membrane hyperpolarization that was persistent and did not recover during the 20 min after the removal of DA. To determine the nature and specificity of G protein coupling, we have attempted to reconstitute DA responses in PTX-treated cells by dialysis with specific α subunits of bovine brain G proteins. [Supported by NIH grant NS-18788.]

10. Bradykinin-induced Currents in Rat Dorsal Root Ganglion Neurons and F-11 Cells Are Insensitive to Pertussis Toxin D. S. MCGEHEE* and G. S. OXFORD, *Department of Physiology, University of North Carolina, Chapel Hill, North Carolina*

Bradykinin (BK) is a potent stimulator of pain in vivo. We have attempted to explore the mechanism of this stimulus in dissociated cultures of sensory neurons. Cultured rat dorsal rat ganglion (DRG) neurons will respond to BK with a transient inward cation current (10–60 pA) that desensitizes markedly. Whole-cell patch-clamp techniques were used to measure BK responses from DRG neurons dissociated from rat pups aged E15 to day 1 postnatal. Cells with smaller sized soma (15–30 μm diam) were preferentially chosen for study and ~50% of these responded to BK (10–1,000 nM). Ion replacement studies have shown that the BK-induced current can be carried by Na^+, Li^+, or Cs^+, but not by large impermeant species such as NMG^+, or TMA^+. Varying the chloride reversal potential had no effect on the response. In standard recording solutions ramp voltage-clamp analyses have shown that the BK current reverses near 0 mV. These data have led us to propose that the activation of a nonselective cation conductance is the mechanism of BK-induced excitation. The mouse neuroblastoma × rat DRG hybrid cell line, F-11, responds to BK with a biphasic response, much like the NG108-15 cell line as reported by Brown et al. (1987). The responses differ, however, in that the second phase of the NG108-15 response represents a conductance decrease, whereas the response in DRG neurons and in F-11 cells results from a conductance increase that reverses near 0 mV. Ewald et al. (1989) have recently reported that pretreatment of DRG neurons with pertussis toxin (PTX) blocks a BK-induced inhibition of whole-cell calcium currents. Inhibition of I_{Ca} was restored by inclusion of the α subunit of any one of three G proteins in the recording electrode. We have observed that in contrast to the I_{Ca} effects, overnight pretreatment with PTX (350 ng/ml) had no obvious effect on BK-induced excitation via the nonselective cation conductance. This may be further evidence that the BK receptor can act through more than one G protein, some of which may be PTX-insensitive. [Supported by NIH grant NS-23804.]

11. Calcium Channel Modulation by G Proteins in Marine *Paramecium.* JUAN BERNAL* and BARBARA EHRLICH, *Departments of Medicine and Physiology, University of Connecticut Health Center, Farmington, Connecticut*

We are interested in the mechanism by which the influx of calcium via calcium channels is modulated and have chosen to use the *Paramecium* as a model system because *Paramecium* is the most primitive organism that has voltage-dependent calcium channels. Previously we reported that the

backward swimming behavior of *Paramecium*, which is correlated with the magnitude of calcium currents, was increased more than sevenfold by incorporating compounds that activate G proteins (McIlveen and Ehrlich. 1988. *Biophys. J.* 53:21*a*). Subsequently we found that the duration of the calcium action potential increased 500% after GTPγS was pressure-injected into the cell while GDPβS reduced the calcium signal (Bernal and Ehrlich. 1989. *Biophys. J.* 55:39*a*). We report here the effects of GTPγS and GDPβS on isolated inward calcium currents in intact cells. To isolate the calcium current, the cells were superfused in the following solution (in millimolar): 125 TEA-Cl, 10 CsCl, 5 4-AP, 5 2-3-diaminopyridine, 15 $CaCl_2$, 10 MOPS, pH 7.3. The compounds of interest were injected into the cell by pressure with fast green as a dye indicator. To evaluate the effects of G protein-related compounds on the calcium currents three criteria were used: (*a*) stable calcium currents before the injection, (*b*) after the injection, the holding current must be stable and the leakage current must not change, and (*c*) it must be observed that the cell is stained after the injection to be sure that the drug is in the cell. In 11 experiments injection of GTPγS, which activates G proteins, enhanced the magnitude of the calcium current 49.5% (from 20 to 90%). In 7 experiments injection of GDPβS, which inactivates G proteins, reduced the calcium current 69.2% (from 28 to 92%). These results support our hypothesis that G proteins modulate voltage-dependent calcium channels in *Paramecium*. [Supported by NIH grant GM-39021-01. B. E. Ehrlich is a Pew Scholar in the Biomedical Sciences.]

12. Activation of Acetylcholine-dependent K Channels in the Absence of Agonist and G Nucleotides

HEIN HEIDBUCHEL* and EDWARD CARMELIET, *Labo voor Electrofysiologie, K.U. Leuven, Leuven, Belgium*

Acetylcholine (ACh) is known to open a specific class of atrial inwardly rectifying K channels (γ ± 40 pS in symmetrical isotonic K^+; τ_o of ±1.5 ms) by means of the intermediate action of a G protein, G_K. We made inside-out patches on atrial cells of the guinea pig with a pipette solution containing in millimolar: 158 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, at pH 7.3 and a bathing solution of the following composition: 128 K-aspartate, 20 KCl, 0.5 or 5.7 free $MgCl_2$, 5.5 glucose, 1 EGTA, 5 HEPES, 4 ATP, at pH 7.3. Under these conditions (absence of GTP_i and ACh_o), the activity of inwardly rectifying K channels with properties of ACh-sensitive K channels (e.g., γ 39 ± 0.9 pS and τ_o 2.1 ± 0.3 ms), spontaneously increased within 0.5–3 min in 121 of 123 patches. The NP_o increased from 0.4 ± 0.1% to 119 ± 18% (n = 14), reaching a maximum after 5–10 min. Remarkably, the channel activity could be inhibited by adding GTP or GDP to the bathing solution. The threshold was 10–100 nM for GDP (n – 3) and 0.1–1 μM for GTP (n = 7). Maximal block, with return to the preactivated opening behavior, occurred with 1–10 μM for both and was reversible after washout of the G nucleotides. Washing of GTPγS, on the other hand, resulted in a persistent activation of the same channels: application of GDP or GTP after GTPγS was without blocking effect. The activation in the absence of ACh and G nucleotides was dependent on ATP (or a contaminant in its preparation) at the intracellular side (threshold of 1 μM, maximum at 0.1–1 mM, n = 5) and this stimulatory effect was fully reversible (n = 35). ATP preparations of Sigma Chemical Co. (A 5394 and 7894; St. Louis, MO) and Boehringer Mannheim (519987; Indianapolis, IN) were equally effective. The activation was comparable in 0.5 or 5.7 mM free Mg^{2+}. Deferoxamine (0.5 mM) did not interfere with the phenomenon, excluding contaminating Al^{3+} to be responsible. ADP could mimic neither the stimulatory ATP effect, nor the blocking GDP effect (n = 3). In contrast, GTPγS activation was not ATP dependent (n = 5). These results indicate that under the described conditions ATP preparations activate ACh-sensitive K channels in inside-out patches of guinea pig atrial cells and that this activation is blocked by the addition of micromolar concentrations of GDP or GTP.

13. A G Protein, G_{i-3}, Regulates a Chloride Channel in Renal Cortical Collecting Duct Cells

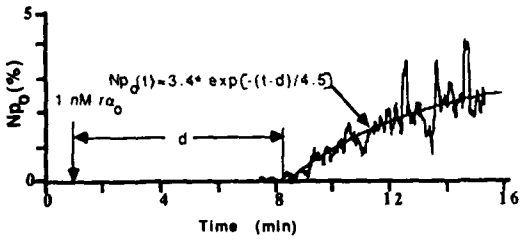
ERIK M. SCHWIEBERT,* DOUGLAS B. LIGHT, and BRUCE A. STANTON, *Department of Physiology, Dartmouth Medical School, Hanover, New Hampshire*

We examined whether G proteins regulate chloride (Cl^-) channels in the apical membrane of rabbit cortical collecting duct (CCD) cells in culture. Primary cultures of CCD cells isolated by

immunodissection and immortalized by transfection with an SV40/adenovirus-2 complex were provided by Arend et al. (1989. *Am. J. Physiol.* 256:In press). Flow cytometric analysis showed that >95% of the cells bound peanut lectin agglutinin. Patch-clamp studies were conducted on inside-out patches of the apical membrane of CCD cells grown on basement membrane–covered glass coverslips. We observed a Cl^- channel with a single-channel conductance of 308 pS; $P_{Cl^-}:P_{Na^+}$ was 9:1. The channel was inhibited by DIDS (4,4'-diisothiocyanaostilbene-2,2'-disulfonic acid) and NPPB (5-nitro-2-[3-phenylpropylamino]-benzoic acid). GTPγS (10^{-4} M) in the bath increased the channel open probability (P_o) from 0.04 to 0.31 ($n = 5$, $P < 0.005$). GTP (10^{-4} M) raised P_o from 0.02 to 0.34 ($n = 7$, $P < 0.01$). In contrast, GDPβS (10^{-4} M) reduced P_o from 0.39 to 0.12 ($n = 6$, $P < 0.05$) and pertussis toxin (PTX; 100 ng/ml) lowered P_o from 0.56 to 0.18 ($n = 5$, $P < 0.01$). In PTX-treated patches, exogenous α_{i-3}^* increased P_o in a dose-dependent manner (2 pM increased P_o to 56% of pre-PTX values, 20 pM increased P_o to 113% of pre-PTX values, and 200 pM increased P_o to 151% of pre-PTX values). α_{i-3}^* also increased P_o in non–PTX-treated inside-out patches. Conclusion: the α_{i-3} subunit of G_i regulates a Cl^- channel in the apical membrane of renal CCD cells. Because GTPγS activation of the channel was observed in the presence of H8 (a protein kinase inhibitor), the G protein does not activate the channel via a protein kinase. The physiological significance of the Cl^- channel and its regulation by G_i is under investigation.

14. Kinetics of Activation of the Cardiac Muscarinic K^+ Channel by Native and Recombinant α Subunits of G Proteins CHAYA JOSHI,* MAURINE LINDER,* DONGHEE KIM, ALFRED G. GILMAN,* and DAVID E. CLAPHAM, *Departments of Physiology and Biophysics and Pharmacology, Mayo Foundation, Rochester, Minnesota; and Department of Pharmacology, University of Texas, Southwestern Medical Center, Dallas, Texas*

The GTP-dependent muscarinic K^+ channel ($i_{K.ACh}$) is activated by both α and βγ subunits of G proteins applied to the intracellular surface of atrial cells. Experiments were performed (blind) to test purified recombinant α subunits ($r\alpha_o$, $r\alpha_{i1}$, $r\alpha_{i2}$, and $r\alpha_{i3}$ synthesized in *Escherichia coli*) for their ability to activate $i_{K.ACh}$ in inside-out patches from neonatal rat atrial cells. Briefly, all the recombinants, including $r\alpha_o$, activated the channel with similar concentration thresholds. Of the patches exposed to 100 pM, 0–23% (2 of 9, 1 of 12, 0 of 4, and 3 of 13 for $r\alpha_o$, $r\alpha_{i1}$, $r\alpha_{i2}$, and $r\alpha_{i3}$) were activated while 50–72% of the patches (7 of 13, 12 of 18, 4 of 8, and 8 of 11 for $r\alpha_o$, $r\alpha_{i1}$, $r\alpha_{i2}$, and $r\alpha_{i3}$) exposed to 1 nM were activated. By comparison, 45% of the patches exposed to picomolar concentrations (5 of 11 patches at 10 pM) of native α subunits (α_{39} and α_{40}) were activated (Logothetis et al. 1988. *Proc. Natl. Acad. Sci.* 85:5814). The channel activity of each patch was fitted by the function, $NP_o(t) = A^* \exp[-(t - d)/\tau]$ (Fig: N = number of channels in patch, P_o = open probability, d = delay between subunit application and start of activation). Each patch ($n > 200$) was exposed to a single concentration of subunit. We will summarize and compare the dose dependence of d, τ, and A for the recombinant and native subunits. The absence of a post- and/or cotranslational modification of recombinant α subunits may explain their relative ineffectiveness in activating the channel compared to native subunits. [Supported by ACS grant BC555K and NIH grants GM-34497 to A.G.G. and HL-41303 to D.E.C.]



15. GTP-γS Potentiates Carbamylcholine-induced Gap Junction Closure in Pancreatic Acinar Cells R. SOMOGYI and H.-A. KOLB, *Faculty of Biology, University of Konstanz, D-7750 Konstanz, Federal Republic of Germany*

The gap junctional conductance (g_j) between pairs of isolated murine pancreatic acinar cells, was analyzed using the double whole-cell patch-clamp technique. Under conditions of stable coupling ($g_j \sim 50$–300 nS), superfusion by carbamylcholine (>1 μM CCh) caused a transient incomplete reduction of the g_j. If GTP-γS (≥10 μM) was added to the pipette solution, CCh induced total electrical uncoupling ($g_j < 3$ pS) in all experiments with a reproducible time course. The lag phase of CCh action decreased with increasing GTP-γS concentration. Application of GTP-γS (500 μM) in the absence of CCh did not influence coupling. If GDP-βS was substituted for GTP-γS no potentiation of the CCh-induced gap junctional channel closure could be observed. Simultaneous addition of polymyxin B suppressed the GTP-γS–induced uncoupling. An uncoupling mechanism based on a G protein-mediated increase of free cytoplasmic calcium could be ruled out. We have previously shown that an increase of protein kinase C activity leads to closure of these gap junction channels and that uncoupling could be suppressed by polymyxin B (Somogyi et al. 1989. *J. Membrane Biol.* 108:In press). Our results are in accord with a model proposing that CCh-induced electrical uncoupling is mediated by a G protein-dependent activation of protein kinase C.

16. Pertussis Toxin Blocks the Hyperpolarization and Reduction in Intracellular Free Calcium Produced by Dopamine or Gamma-Aminobutyric Acid in Rat Melanotrophs P. STEPHEN TARASKEVICH and WILLIAM W. DOUGLAS, *Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut*

The melanotrophs of the mammalian pars intermedia are directly innervated by neurons of central origin. A dopaminergic and GABAergic component of this innervation have been identified, and dopamine (DA), acting through D2 receptors, and gamma-aminobutyric acid (GABA), acting through $GABA_B$ receptors, inhibit spontaneous electrical activity, lower the basal intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), and decrease hormone output in melanotrophs. Here we report effects of DA and GABA, acting through such receptors, on membrane potential and show that these effects as well as those on $[Ca^{2+}]_i$ are blocked by pertussis toxin (PTX). Melanotrophs were dispersed from rat neurointermediate lobes and cultured 17–24 h in Ham's F-10. Changes in membrane potential were monitored with bis-oxonol (200 nM) and $[Ca^{2+}]_i$ was measured with either fura-2 or indo-1. Fluorescence measurements were made from suspensions of cells stirred in a microcuvette that was held in a thermostated (37°C) holder. All responses were recorded in tetrodotoxin (5 μM) to suppress spontaneous Na spike activity. DA (1 nM–1 μM) induced a hyperpolarization the magnitude of which was dose dependent. This effect was mimicked by the specific D2 agonist LY 171555 (1 μM) and was blocked or reversed by the D2 antagonist sulperide (4 μM) but not the D1 antagonist SCH 23390 (4 μM). GABA (100 μM) in the presence of the $GABA_A$ receptor antagonist bicuculline methiodide (BMI, 200 μM), also caused a hyperpolarization which was mimicked by the $GABA_B$ receptor agonist (−)baclofen (50 μM) but not by the $GABA_A$ receptor agonist muscimol (50 μM). Pretreatment of the melanotrophs with PTX (10–100 ng/ml) for 17–24 h abolished the hyperpolarization produced by DA or GABA (in BMI) and also blocked the decrease in $[Ca^{2+}]_i$ caused by these agents (see Nemeth et al. 1988. *J. Gen. Physiol.* 92:9*a*), but did not block the changes in membrane potential or $[Ca^{2+}]_i$ produced by excess K^+ (50 mM). [Supported by NIH grant NS-09137.]

17. Carbachol Increases Basolateral K^+ Permeability in T84 Cells by Increasing Cell [Ca] HERBERT CHASE, JR. and SHIRLEY WONG,* *Department of Medicine, Columbia University, New York, New York*

Carbachol stimulates transepithelial chloride secretion in a variety of tissues. Though intracellular free [Ca] ($[Ca]_i$) is known to play an important role in carbachol's action, it is not known where calcium acts. We examined the possibility that calcium increases the potassium permeability of the

basolateral membrane by simultaneously measuring $[Ca]_i$ and the basolateral K^+ permeability in T84 cell monolayers, which secrete chloride and are hormonally responsive. $[Ca]_i$ was measured using fura-2, fluorescence microscopy, and simple video imaging. To assess changes in basolateral K^+ permeability, we measured the short circuit current (I_{sc}) in the presence of luminal amphotericin and a transepithelial mucosa-to-serosa K^+ gradient (Germann et al. 1986. *J. Gen. Physiol.* 88:237). We found that carbachol increases the basolateral K^+ permeability and that calcium plays a major role in carbachol's action. First, carbachol caused I_{sc} and $[Ca]_i$ to increase in parallel. I_{sc} rose from 161 ± 52 μA to a peak of 273 ± 88 while $[Ca]_i$ rose from 104 ± 17 nM to a peak of 342 ± 74. Approximately 5 min after the peak response both I_{sc} and $[Ca]_i$ returned to values only slightly above control. Second, the carbachol-induced increase in I_{sc} was correlated significantly to the increase in $[Ca]_i$. Third, carbachol's action on transport was significantly reduced by blunting the calcium spike with the intracellular calcium buffer BAPTA. Last, the carbachol-induced parallel changes in I_{sc} and $[Ca]_i$ could be mimicked by increasing $[Ca]_i$ with ionomycin. Thus, the carbachol-induced increase in $[Ca]_i$ appears to be necessary for the increase in I_{sc}. It is not clear, however, if carbachol's action is solely the result of an increase in $[Ca]_i$. Increasing $[Ca]_i$ with ionomycin, though causing I_{sc} and $[Ca]_i$ to increase in parallel, failed to increase I_{sc} to the levels observed with carbachol. These results suggest that carbachol may stimulate a second process that enhances calcium's action on the K^+ permeability of the basolateral membrane. [Supported by NIH DK-39154.]

18. Oscillations of Intracellular Ca^{2+} Induced by Cholinergic Activation in a Human Secretory Epithelium (T_{84}) DANIEL C. DEVOR,* ZAHUR AHMED,* and MICHAEL E. DUFFEY, *Department of Physiology, School of Medicine, State University of New York, Buffalo, New York*

We have previously demonstrated that the cholinergic agonist carbamyl-choline (carbachol) induces oscillations of membrane K^+ conductance in isolated T_{84} cells. These oscillations are dependent on the release of Ca^{2+} from intracellular stores. (Devor et al. 1989. *FASEB J.* 3:A1149) In the present study, we used fluorescence microscopy and the Ca^{2+}-sensitive dye, fura-2, to determine whether oscillations of intracellular free Ca^{2+}, $[Ca^{2+}]_i$, may underlie these K^+ conductance oscillations in isolated cells and monolayers. Resting $[Ca^{2+}]_i$ in isolated cells averaged 58 ± 7 nM (±SEM, $n = 44$). Exposure of these cells to carbachol (100 μM) caused a rapid rise in $[Ca^{2+}]_i$ of 173 ± 16 nM that was blocked by atropine (10 μM). This initial peak was followed by a gradual decline and oscillations of $[Ca^{2+}]_i$ in 70% of the responding cells. When the bath was made Ca^{2+} free (with 0.5 mM EGTA) the response to carbachol was the same ($n = 13$). As the dose of carbachol was decreased the magnitude of the initial $[Ca^{2+}]_i$ peak decreased, the percent of responding cells decreased, and latency of the response increased. In addition, the frequency of $[Ca^{2+}]_i$ oscillations decreased as the dose of carbachol decreased. In individual cells of a monolayer carbachol caused a rapid rise in $[Ca^{2+}]_i$ of 149 ± 19 nM ($n = 8$) that gradually declined to a steady-state plateau that was 48 ± 9 nM higher than the resting value, but oscillations were never seen. Identical responses were seen in large groups of cells of the same monolayers. These results demonstrate that carbachol causes a rapid increase in $[Ca^{2+}]_i$ in isolated T_{84} cells, followed by oscillations that are related to interactions between cytoplasmic Ca^{2+} and intracellular Ca^{2+} stores and not Ca^{2+} influx. The absence of oscillations in the intact monolayer may be due to the formation of gap junctions or other features of cell differentiation which remain to be elucidated. [Supported by NIH grants HL-28542 and NS-27144.]

19. Mechanisms Responsible for Mechanical Abnormalities in Hypertrophied and Failing Myocardium CYNTHIA L. PERREAULT,* OSCAR H. L. BING,* WESLEY W. BROOKS,* BERNARD J. RANSIL,* and JAMES P. MORGAN, *Department of Medicine, Harvard Medical School, Boston, Massachusetts*

We tested the hypothesis that contractile dysfunction seen with hypertrophy (H) and failure (CHF) is due to abnormal intracellular calcium handling and/or altered myofilament Ca^{2+} sensitiv-

ity. We studied muscles from spontaneously hypertensive rats (SHR). CHF was diagnosed by effusions, left atrial thrombi, and right ventricular hypertrophy (RVH). Six Wistar-Kyoto rats (WKY) 18–24 mo were age-matched controls. Six SHRs developed CHF (SHR-F) and seven did not (SHR-NF). Left ventricular (LV) papillary muscles were macroinjected with aequorin, a bioluminescent Ca^{2+} indicator (Kihara and Morgan. 1988. *J. Gen. Physiol.* 92:47*a*) and intracellular Ca^{2+} ($[Ca^{2+}]_i$) transients were recorded. Active tension was reduced in SHR-NF (1.9 ± 0.4) and SHR-F (1.5 ± 0.5) compared with WKY (3.2 ± 0.4) (g/mm^2; $P < 0.05$) and time to peak tension was prolonged in SHR-NF and SHR-F. These mechanical abnormalities were accompanied by a prolonged $[Ca^{2+}]_i$ transient. Myofilament Ca^{2+} sensitivity was investigated using saponin-skinned (250 μg/ml) LV trabeculae and RV strips. The muscles were activated by Ca^{2+} buffers (10^{-8}–10^{-4} M). Data were plotted as pCa($-\log[Ca^{2+}]$M) vs. isometric force and values of half-maximal force (pCa_{50}) and maximal Ca^{2+}-activated force (F_{max}) were generated. In the normotensive WKY, we found a decreased Ca^{2+} sensitivity (5.65 ± 0.25) in the RV compared to LV (6.01 ± 0.26). In the LV there was no change in Ca^{2+} sensitivity in H and CHF. In the RV, the Ca^{2+} sensitivity was increased from 5.65 ± 0.25 (control WKY) to 6.09 ± 0.26 (SHR-F). The RV may be able to adapt to an increase in pressure caused by H and CHF by increasing Ca^{2+} sensitivity whereas the LV is not able to modify its myofilament Ca relationship in response to changing physiologic conditions. Conclusion: the contractile abnormalities seen with H and CHF are probably not due to altered Ca^{2+} sensitivity at the myofilament level but may be caused by abnormal intracellular calcium handling.

20. Thapsigargin, but Not Caffeine, Blocks the Ability of Thyrotropin-releasing Hormone to Release Ca^{2+} from an Intracellular Store in GH_4C_1 Pituitary Cells GREG J. LAW, JONATHAN A. PACHTER, OLE THASTRUP, and PRISCILLA S. DANNIES, *Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut* (Sponsor: P. S. Taraskevich)

Thapsigargin (TG) stimulates release of Ca^{2+} from an intracellular store in platelets (Thastrup et al. 1987. *Biochim. Biophys. Acta.* 927:65) and neuroblastoma cells (Jackson et al. 1988. *Biochem. J.* 253:81). We investigated the effects of TG on GH_4C_1 pituitary cells. 1 μM TG caused a rise in cytosolic Ca^{2+} that was followed by a lower sustained elevation of $[Ca^{2+}]_c$ as measured by indo-1. TG induced similar changes in the presence of 1 μM nimodipine, a Ca^{2+} channel antagonist. TG did not cause a detectable increase in $^{45}Ca^{2+}$ influx but did stimulate the rate of $^{45}Ca^{2+}$ efflux. TG has no effect on inositol phosphate accumulation. In medium with low Ca^{2+} concentration, TG caused a transient increase in $[Ca^{2+}]_c$. Therefore, the initial rise in $[Ca^{2+}]_c$ does not require extracellular Ca^{2+}. Thyrotropin-releasing hormone (TRH) mobilizes Ca^{2+} from an IP_3-sensitive store (Gershengorn et al. 1984. *J. Biol. Chem.* 259:10675). Pretreatment with TG blocked the ability of TRH to cause a $[Ca^{2+}]_c$ transient. It is unlikely that TG reduces the Ca^{2+} signal by stimulating Ca^{2+} efflux because the TRH-induced stimulation of $^{45}Ca^{2+}$ efflux was also blocked. The block of TRH-induced Ca^{2+} mobilization was not caused by a block at the receptor level because TRH stimulation of IP_3 formation was not affected by TG. In medium with low Ca^{2+} concentration, TG blocked the rise in $[Ca^{2+}]_c$ by TRH, but TRH did not completely block the rise in $[Ca^{2+}]_c$ caused by TG. Ca^{2+}-induced Ca^{2+} release does not account for the action of TG on the TRH spike in $[Ca^{2+}]_c$ because BAY K 8644 (1 μM), which causes a sustained rise in $[Ca^{2+}]_c$, did not block Ca^{2+} release caused by TRH. In addition, 10 mM caffeine, which releases Ca^{2+} from intracellular stores in other cell types, caused a $[Ca^{2+}]_c$ spike in GH_4C_1 cells, but had no effect on a subsequent $[Ca^{2+}]_c$ spike induced by TRH. We conclude TG selectivity blocks the ability of IP_3 to release $[Ca^{2+}]_c$, either by discharging the IP_3-releasable pool, or by blocking the ability of IP_3 to release Ca^{2+}.

21. The Effect of an Endogenous Factor on the Isolated Ca^{2+} Release Channel of the Sarcoplasmic Reticulum ANNEGRET HERRMANN-FRANK,* GERHARD MEISSNER,* and ERIC ROUSSEAU,* *Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina; and Department of Physiology and Biophysics, University of Sherbrooke, Sherbrooke, Canada* (Sponsor: Gerry S. Oxford)

From caffeine-treated "oscillating" skeletal muscle fibers, a low molecular material is released that has been found to generate subthreshold sarcomeric oscillations and long-lasting repetitive cyclic contractions in isolated skinned skeletal muscle fibers (Herrmann. 1987. *Pflüg. Arch.* 408:R80). In the experiments presented here, the effect of this material was tested directly on the isolated Ca^{2+} release channel of the sarcoplasmic reticulum (SR). In one approach, $^{45}Ca^{2+}$ efflux behavior was determined in passively loaded heavy density SR vesicles isolated from rabbit skeletal muscle, according to the procedure of Meissner (1984. *J. Biochem.* 259:2365). The effect of the material released from oscillating muscle fibers was tested in a medium containing a low concentration of free Ca^{2+} (0.1 μM), 1 mM free Mg^{2+}, and 5 mM AMP. The material induced a small, but significant 1.6–1.8-fold increase in the $^{45}Ca^{2+}$ efflux rate. This increase was comparable to the stimulating effect of a release medium containing 0.5–1.0 mM caffeine. The action of the material was also tested at the single-channel level by fusing heavy SR vesicles into Mueller-Rudin planar lipid bilayers, as described by Smith et al. (1988. *Meth. Enzymol.* 157:480). Addition of the active material to the cytoplasmic (*cis*) side of the Ca^{2+} channel increased the open probability two- to fourfold, whereas the mean current amplitude remained unchanged. The effect was more pronounced in the presence of low free *cis* Ca^{2+} (0.5 μM). However, no voltage-dependent effect was observed. The "drug"-induced potentiation of channel activity was reduced by Mg^{2+} or ruthenium red on the *cis* side. Together with the data obtained from skinned fiber experiments, these results suggest that the factor released from caffeine-treated skeletal muscle fibers acts directly on the Ca^{2+} release channel by enhancing SR Ca^{2+} release. [Supported by SFB 114 (DFG) and USPHS grant AR-18687.]

22. Effects of Thrombin on Calcium Transport in Cultured Cardiac Myocytes WALTER CHIEN,* RAJENDRA MOHABIR,* LAWRENCE L. LEUNG,* WILLIAM T. CLUSIN. *Cardiology Division, Stanford University School of Medicine, Stanford, California*

Thrombin is known to stimulate IP_3 formation in several cell types, and to elevate cytosolic calcium, $[Ca^{++}]_i$. There is an associated positive ionotropic effect in the heart. To determine the mechanism of the $[Ca^{++}]_i$ increase, we studied the effects of thrombin in chick embryonic heart cells loaded with the $[Ca^{++}]_i$ indicator, indo-1 AM. Thrombin (1 U/ml) increased peak systolic $[Ca^{++}]_i$ from 573 ± 61 to 1,133 ± 120 nM, and end-diastolic $[Ca^{++}]_i$ from 303 ± 38 to 632 ± 55 nM ($P < 0.002$). This effect was blocked by the pentapeptide thrombin inhibitor "PPACK." Thrombin increased beat frequency from 108 ± 9 to 121 ± 9 /min ($P < 0.0005$), and increased contraction strength by 7% ($P < 0.005$). Action potential duration also increased. The thrombin-induced increase in $[Ca^{++}]_i$ was not blocked by TTX (30 μM) or verapamil (50 μM), but was abolished by 6 h pretreatment with pertussis toxin (100 ng/ml). A thrombin-induced $[Ca^{++}]_i$ increase could still be demonstrated in zero extracellular Ca^{++} (with EGTA), and this effect was potentiated by 10 mM Li^+, which inhibits breakdown of inositol phosphates. Thrombin also increased Ca^{++} influx across the surface membrane. This was shown by thrombin-induced entry of Mn^{++}, which quenches indo-1 fluorescence at all wavelengths, including the Ca^{++}-insensitive isosbestic wavelength, 430 nm. Thrombin-induced Mn^{++} entry was not prevented by verapamil (50 μM), which indicates that L-type calcium channels are not involved. We conclude that thrombin promotes calcium entry and release in embryonic heart cells, even when they are rendered quiescent by sodium or calcium channel blockade. Both processes may be coupled to the receptor by pertussis-sensitive G proteins, which may act by increasing IP_3.

23. Sustained Ca^{2+} Entry and $[Ca^{2+}]_i$ Elevation in Carbachol- and $NaF/AlCl_3$-stimulated Rat Parotid Acini LAWRENCE M. MERTZ,* VALERIE J. HORN,* BRUCE J. BAUM,* and INDU S. AMBUDKAR, *Clinical Investigation and Patient Care Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland*

Muscarinic/cholinergic stimulation of the rat parotid gland results in a Ca^{2+}-dependent biphasic increase in fluid secretion rate. In the first phase, there is an increase in the rate of K^+ and Cl^- efflux, which is concurrent with increases in inositol polyphosphate production and a mobilization of intracellular Ca^{2+} ($[Ca^{2+}]_i$) stores. The later phase is represented by a lower rate of sustained fluid

secretion which is dependent upon extracellular Ca^{2+} ($[Ca^{2+}]_o$) and, by implication, a Ca^{2+} influx mechanism which remains largely uncharacterized. We have examined the characteristics of carbachol-stimulated Ca^{2+} entry in preparations of quin-2–loaded, collagenase-dispersed rat parotid acini. Carbachol (10 μM) evokes a rapid (5 s), transient (300 s), three- to fourfold increase (at peak) in cytosolic Ca^{2+} levels, which is followed by a smaller sustained rise in cytosolic Ca^{2+}. This "sustained phase" is receptor specific (i.e., atropine sensitive) and dependent upon $[Ca^{2+}]_o$. If, during this rise, additional Ca^{2+} is added to the extracellular medium, a further Ca^{2+} gradient-dependent increase in $[Ca^{2+}]_i$ occurs which is carbachol dose dependent. We also examined the effects of NaF (10 mM)/$AlCl_3$ (10 μM) on $[Ca^{2+}]_i$ in rat parotid acini. After a slow, transient intracellular release of Ca^{2+}, NaF/$AlCl_3$ induces a large sustained increase in $[Ca^{2+}]_i$. As in carbachol-treated cells, this sustained rise is dependent upon $[Ca^{2+}]_o$ and can be increased further if $[Ca^{2+}]_o$ is elevated before or during this rise. These data indicate that during prolonged periods of carbachol stimulation, there is a mechanism of Ca^{2+} entry into parotid acinar cells, which may support sustained fluid secretion. Moreover, since we observe Ca^{2+} entry in acini stimulated with NaF/$AlCl_3$, we suggest that it is likely to be controlled either directly or indirectly via G protein activation.

24. Tenidap (CP-66,248): An Inhibitor of Receptor-operated Calcium Channels in Mast Cells? PATRICIA L. CLEVELAND,* GORDON C. YANEY,* HENRY J. SHOWELL,* and CLARE FEWTRELL, *Department of Pharmacology, Cornell University, Ithaca, New York; and Department of Immunology and Infectious Diseases, Pfizer Central Research, Groton, Connecticut*

The antigen-induced cross-linking of immunoglobulin E receptors on the surface of tumor mast cells leads to a secretory response that is accompanied by changes in intracellular free ionized calcium. It is now clear that both release of Ca^{2+} from intracellular stores and influx of Ca^{2+} across the plasma membrane contribute to the Ca^{2+} responses that are required for the initiation of secretion. The new antiinflammatory agent tenidap (5-chloro-2,3-dihydro-2-oxo-3-[2-thienylcarbonyl]-indole-1-carboxamide) shows promise as an inhibitor of Ca^{2+} influx. In tumor mast cells, tenidap inhibits the antigen-induced secretion of [^{3}H]-serotonin and β-hexoaminidase with an IC_{50} of ~10 μM. Ca^{2+} influx, as measured by both ^{45}Ca flux studies and fura-2 fluorescence, is also inhibited at similar concentrations. Moreover, tenidap inhibits antigen-induced depolarization measured by bis-oxonol fluorescence. This depolarization has been shown to be an indicator of Ca^{2+} influx in tumor mast cells (Mohr and Fewtrell. 1987. *J. Immunol.* 138:1564) and so this result also suggests that Ca^{2+} influx is inhibited by tenidap. Phosphoinositide breakdown, measured by the incorporation of [^{3}H]inositol into inositol phosphates, is only partially inhibited by tenidap. Consistent with this, tenidap appears to have little effect on antigen-stimulated efflux of ^{45}Ca, which is thought to reflect inositol 1,4,5-trisphosphate-stimulated release of Ca^{2+} from intracellular stores. However, the compound appears to increase spontaneous efflux of ^{45}Ca in unstimulated cells. In contrast to its effects in tumor mast cells, tenidap does not appear to inhibit KCl-induced insulin secretion in RINm5F cells, a rat insulinoma cell line in which insulin secretion is tightly coupled to the influx of Ca^{2+} through L-type voltage-dependent Ca^{2+} channels (Wollheim and Pozzan. 1984. *J. Biol. Chem.* 259:2262). These results suggest that tenidap may prove useful in investigating the Ca^{2+} channels of nonexcitable cells. [Supported by grants from the Cornell Biotechnology Program.]

25. Characterization of Latency in the $[Ca^{2+}]_i$ Response to IgE Receptor Cross-linking in Tumor Mast Cells PAUL J. MILLARD, TIMOTHY A. RYAN,* LI-MING SU,* WATT W. WEBB, and CLARE FEWTRELL, *Departments of Pharmacology and Physics and School of Applied and Engineering Physics, Cornell University, Ithaca, New York*

We have used digital video imaging microscopy of fura-2 fluorescence to monitor changes in intracellular free ionized calcium ($[Ca^{2+}]_i$) that occur upon cross-linking of IgE receptors on the surface of mast cells. Our previous results have shown that the rapid increase in cytosolic calcium in stimulated rat basophilic leukemia (RBL) cells of the 2H3 clonal line is preceded by lag times of varying duration (Millard et al. 1988. *Proc. Natl. Acad. Sci.* 85:1854) and that the average length of

this latency is inversely related to antigen concentration. We have examined several possible reasons for the wide distribution of latencies. RBL-2H3 cells were recloned by the limiting dilution method and the distribution of latencies of individual cells in the population were analyzed to determine whether genetic divergence, which frequently occurs in continuously passaged cell lines, could have given rise to this wide variation in latency. To see whether the typical distribution of cells in different stages of mitosis could affect the distribution of latencies, measurements of $[Ca^{2+}]_i$ were also performed using mitotically synchronized cells that had been selected by nocodozole pretreatment followed by a mitotic shake-off procedure. Neither recloning nor cell cycle synchronization dramatically affected the distribution of latencies of the calcium responses. Studies have been carried out using different cross-linking ligands at varying surface receptor densities to carefully characterize the effect on the latency of the response. We are currently studying the correlation between the latencies that precede responses in the same cell after sequential stimulation with the same or different ligands to determine whether latency is an intrinsic property of each individual cell. [Supported by grants from the NSF DCB-8702584, DIR-8716854 (C.M.S.F.), and DMB-8609084 (W.W.W.); the NIH GM-33028 (W.W.W.) and in part by grants from the Cornell Biotechnology Program.]

26. Receptor-activated Calcium Entry in Exocrine Cells Does Not Occur Via Agonist-sensitive Intracellular Pools: the "Capacitative Model" Revised TREVOR J. SHUTTLEWORTH,* *Department of Physiology, University of Rochester School of Medicine and Dentistry, Rochester, New York* (Sponsor: Peter Shrager)

In the majority of exocrine cells, intracellular signalling pathways for ion and fluid secretion involve increases in intracellular calcium ion concentrations ($[Ca^{2+}]_i$) that are comprised of two components, a mobilization of intracellular Ca^{2+} stores, and a sustained entry of Ca^{2+} from the extracellular medium. Current models of this receptor-activated Ca^{2+} entry involve the formation of some form of physical communication between the emptied agonist-sensitive intracellular pools and the plasma membrane. Entry of extracellular Ca^{2+} into the cytoplasm then occurs via the open intracellular pools. This has been described as "capacitative" Ca^{2+} entry. However, in the avian nasal gland, a model exocrine ion-secreting tissue, it can be demonstrated under appropriate conditions that refilling of the intracellular stores after termination of receptor activation is associated with a marked transient increase in $[Ca^{2+}]_i$, suggesting that Ca^{2+} entry during this phase occurs via the cytoplasm rather than directly into the empty pools. Furthermore, the initial rates of increase in $[Ca^{2+}]_i$ during stimulation are faster in conditions where both Ca^{2+} entry and Ca^{2+} release occur (i.e., they are additive). Such data are inconsistent with current models in which the rate of Ca^{2+} entry is determined by the relative "emptiness" of the intracellular pools as, in such a model, the rate of Ca^{2+} entry through the pools cannot exceed the rate of Ca^{2+} release. Thus, Ca^{2+} entry during the initial stimulation phase also appears to be direct into the cytoplasm and not via the intracellular pools. Ca^{2+} entry and Ca^{2+} release must therefore occur via separate pathways operating in parallel, and not in sereis as previously predicted. [Supported by NIH grant GM-40457.]

27. Calcium Transients in Frog Skeletal Muscle Fibers Measured with the Fluorescent "Magnesium" Indicator, Mag-fura-2 M. KONISHI, S. HOLLINGWORTH, and S. M. BAYLOR, *Department of Physiology, University of Pennsylvania, Philadelphia, Pennsylvania; and Department of Physiological Sciences, University of Newcastle upon Tyne, England*

Mag-fura-2 (also called Furaptra by Raju et al. 1989. *Am. J. Physiol.* 256:C540) is listed by Molecular Probes, Inc. (Eugene, OR) as an intracellular magnesium indicator. However, Mag-fura-2 has an affinity for Ca^{2+} about 100-fold higher than that for Mg^{2+} (dissociation constants of 44 μM and 5.1 mM, respectively, in 120 mM KCl, 10 mM NaCl, pH 7.0, 16°C; also see Raju et al. 1989). Thus, for a cell like a muscle fiber, which undergoes a substantial increase in free $[Ca^{2+}]$ during activity, the indicator cannot be expected to provide an accurate estimate of changes in free $[Mg^{2+}]$. In fact, during the twitch of intact single fibers microinjected with Mag-fura-2, there is a readily detected fluorescence change, ΔF, from the indicator (excitation, 420 nm; emission, >495 nm) that appears

to be driven by the myoplasmic free $[Ca^{2+}]$ transient, $\Delta[Ca^{2+}]$, with minimal interference from Mg^{2+}. Interestingly, the ΔF from Mag-fura-2, in contrast to that from fura-2 (cf. Baylor and Hollingworth. 1988. *J. Physiol.* 403:151), appears to track $\Delta[Ca^{2+}]$ with little or no kinetic delay, and also appears to give a more accurate calibration of the amplitude of $\Delta[Ca^{2+}]$. We conclude that Mag-fura-2 is a more useful Ca^{2+} indicator than fura-2 for preparations in which resolution of a large and rapid Ca^{2+} transient is required. [Supported by NIH grant NS-17620.]

28. Effects of Intracellular Dialysis with Nonhydrolyzable GTP Analogues on Exocytosis in Bovine Pituitary Lactotrophs. S. K. SIKDAR, R. ZOREC, W. T. MASON, *Agricultural Research Council Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, United Kingdom; Institute of Pathophysiology, Ljubljana, Yugoslavia*

The secretion of prolactin by lactotrophs of the anterior pituitary gland is affected by the neurohormones dopamine (DA) and thyrotropin-releasing hormones (TRH). Receptors to these hormones are linked to intracellular events via G proteins. There is evidence that the transducing action is indirect, and is mediated by affecting cAMP content, IP_3 turnover, Ca influx, and K^+ channel permeability. However, it is still unknown whether G proteins can directly affect exocytosis independent of other cellular factors. We have investigated this issue by dialyzing single cultured bovine pituitary lactotrophs with nonhydrolyzable GTP analogues to activate G proteins, while simultaneously measuring cell membrane capacitance (C_m) to monitor exocytosis under voltage clamp using a lock-in amplifier incorporated into the patch-clamp amplifier. Exocytosis was promoted in the presence of high intracellular Ca^{2+} as the dialysate ($\simeq$1 μM Ca^{2+}, estimated from 0.5 EGTA and 3.5 Ca-EGTA, and a K_d of 0.15 μM; n = 16). Inclusion of the nonhydrolyzable GTP analogues GTP-γ-S (40 μM, n = 9) and GMP-PNP (100 μM, n = 9) suppressed the exocytotic response, indicating suppression of Ca-dependent exocytosis. The rate of capacitance change indicative of the speed of granule translocation towards the membrane fusion sites was, however, accelerated in the G protein-activated cells. A Ca^{2+}-free solution containing 10 mM EGTA in the dialysate *failed to initiate exocytosis* (n = 28) and 40 μM GTP-γ-S inclusion (n = 5) had no effect. Our results suggest a dual effect of G protein activation on Ca-dependent exocytosis, possibly mediated through different G protein pathways. [Supported by Kabi Vitrum AB, Sweden, and the Wellcome Trust, UK].

29. Single-Channel and Whole-Cell Recordings ot Two Types of Ca^{2+} Currents in Gastric Smooth Muscle Cells: Effects of the Dihydropyridine Bay K 8644. MICHEL B. VIVAUDOU,* JOSHUA J. SINGER, and JOHN V. WALSH, JR., *Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts*

In freshly isolated gastric smooth muscle cells from the toad, *Bufo marinus*, tight-seal, whole-cell recordings (1.8 or 20 mM Ca^{2+} in the bath, 130 CsCl in the pipette) have revealed two types of whole-cell Ca^{2+} currents: (*a*) a rapidly inactivating current activated by small depolarizations and (*b*) a larger, slowly inactivating current that has a higher activation threshold, is susceptible to run-down, and is augmented by acetylcholine or substance P (Vivaudou et al. 1988. *FASEB J.* 2:2497; Clapp et al. 1989. *Pflügers Arch.* 413:565). Brief application of the dihydropyridine agonist Bay K 8644 caused a rapid, reversible increase in the Ca^{2+} current. Recordings of unitary currents in cell-attached patches (90 mM Ba^{2+} in the pipette, 130 K aspartate outside the cells) confirmed the existence of two distinct populations of voltage-activated Ca^{2+} channels. One type (L) had a conductance of 24 pS with an extrapolated reversal potential of 55 mV (n = 24) and was observed only in the presence of Bay K 8644 (5–10 μM in the pipette or applied to the outside of the cell). The other (TN for T or N) had a conductance of 11 pS with an extrapolated reversal of 49 mV (n = 29) and could be recorded in the absence of Bay K 8644. Although most patches contained both types, each type has been recorded in isolation on several occasions. After patch excision, TN channels remained active while L channels quickly ran down, suggesting that the latter underlie the high-threshold whole-cell current. When elicited by short depolarizing pulses (300 ms long every 3 s), L channels showed the typical gating behavior of L channels from other tissues, either failing to open or opening throughout the pulse. With longer pulses (3–5 s long every 18 s) L channels usually

activated and then inactivated within the first 500 ms of depolarization. In some patches, however, L channel activity persisted throughout the seconds-long period of the depolarization. [Supported by NIH DK-31620 and NSF DCB-8511674.]

30. Structure-Function Studies on the $(Na^+ + K^+)$-ATPase. BRUCE KONE,* KUNIO TAKEYASU,* VICTOR LEMAS,* and DOUGLAS FAMBROUGH, *Departments of Biology and Medicine, The Johns Hopkins University, Baltimore, Maryland.*

The sodium pump [$(Na^+ + K^+)$-ATPase] consists of heterodimeric alpha-beta subunit complexes in which most or all aspects of ion transport are associated with the alpha subunit. There are at least three molecular forms (isoforms) of the alpha subunit in mammals and birds. Within a species the isoforms differ at ~20% of their aminoacyl residues. However, each isoform is more highly conserved in evolution: the alpha-1 isoforms of rat and chicken, for example, differ by only ~7%. This suggests that some aspects specific to the function or regulation of each isoform constrain evolutionary divergence. Such aspects might include subtle differences in ion transport. We have been studying sodium pump function by expressing DNAs encoding ouabain-sensitive avian sodium pumps in mouse cells that express an ouabain-resistant alpha-1, and by then monitoring ouabain-sensitive transport. Through this approach we found that ouabain sensitivity is determined by the alpha subunit (Takeyasu et al. 1988. *J. Biol. Chem.* 263:4347). In studies conducted to compare alpha isoform function, mouse cells expressing a chicken beta subunit were transfected with DNA that encodes for chicken alpha subunits, and cell lines expressing each of the three chicken alpha isoforms were selected. Each line expressed high-affinity ouabain-binding sites and ouabain-sensitive ion transport. The major parameters of transport now under analysis include affinities for sodium and potassium ions at intracellular and extracellular sites and affinities for ATP. In this system it will also be possible to look for isoform-specific modulation of transport in response to various influences that act through second messengers. [Supported by NIH grant NS-23241 and NIH Clinical Investigator Award DK-01885.]

31. $[Na]_i$ and $[K]_o$ Determine Apparent Affinity of the Na/K Pump for Ouabain in Cardiac Myocytes JOSEPH R. STIMERS, SHI LIU*, LESLIE A. LOBAUGH, and MELVYN LIEBERMAN, *Department of Cell Biology, Division of Physiology, Duke University Medical Center, Durham, North Carolina*

Voltage-clamp, Na-selective microelectrode (Na-SME) and equilibrium [^{3}H]ouabain binding techniques were used to study two factors ($[Na]_i$ and $[K]_o$) affecting the apparent affinity ($K_{0.5}$) of the Na/K pump for ouabain in cultured embryonic chick cardiac myocytes. Small aggregates (80–120 μm diam) that were voltage clamped at −80 mV and bathed in control solution (5.4 mM K + 1 mM Ba) had a $K_{0.5}$ of 20.6 ± 1.2 μM. Addition of 3 μM monensin, a Na/H ionophore, shifted $K_{0.5}$ to 2.4 ± 0.2 μM. In experiments that combined voltage clamp and Na-SME recordings, we found that $[Na]_i$ was 7.9 ± 1.6 mM in control solution, while in 3 μM monensin $[Na]_i$ increased to 24.1 ± 0.4 mM (converted from a^i_{Na} with activity coefficient of 0.735). Equilibrium [^{3}H]ouabain binding experiments on confluent heart cell cultures in 0.0, 0.5, or 5.4 mM K solutions show a $K_{0.5}$ of 0.56 ± 0.14, 0.43 ± 0.03, and 6.6 ± 0.4 μM, respectively (Lobaugh and Lieberman. 1987. *Am. J. Physiol.* 253:C731–C743). We have simulated these results with a cyclic model of the Na/K pump modified from Chapman et al. (1983. *J. Memb. Biol.* 74:139–153) to include ouabain binding with high affinity to a single conformation of the Na/K pump. With $[Na]_i$ being the only free parameter in the model, the experimentally measured $K_{0.5}$ of 20.6, 6.6, and 2.4 μM in 5.4 mM K correspond to $[Na]_i$ of 7.1, 12.4, and 25.4 mM, respectively. The model predicts that if $[K]_o$ is below 1 mM, the $K_{0.5}$ is relatively insensitive to both $[Na]_i$ and $[K]_o$ and is ~0.7 μM, which is in good agreement with the measured values obtained in 0.0 and 0.5 mM K. In conclusion, the $K_{0.5}$ for ouabain was found to depend on $[Na]_i$ and $[K]_o$, perhaps because these ions shift the distribution of the Na/K pump among its multiple conformations. [Supported by NIH grants HL-27105, HL-17670, HL-07101, and HL-07063.]

32. Effects of Cellular and External Na and K on the Rate of Orthophosphate-promoted Ouabain Binding to Resealed Human Red Cell Ghosts M. GUERRA,* M. STEINBERG,* and P. B. DUNHAM, *Departamento de Fisiologia, Universidad de la Laguna, Tenerife, Canary Islands, Spain; Department of Pharmacology, State University of New York Health Science Center, Syracuse, New York, and Department of Biology, Syracuse University, Syracuse, New York*

Ouabain is thought to bind preferentially to the E_2P-phosphorylated intermediate of Na,K-ATPase. Inhibition of ATP-promoted ouabain binding by external K is explained in terms of the Albers-Post mechanism by stimulation of dephosphorylation of E_2P. Ouabain binding can be promoted by reversal of this hydrolytic step, i.e., phosphorylation by orthophosphate (P_i) and Mg. This reaction might be enhanced by K binding intracellularly. To test this, the rate of ouabain binding promoted by P_i and Mg was measured in resealed human red cell ghosts, which allow cellular and external cation concentrations to be varied independently. Contrary to the prediction, the rate of P_i-promoted binding was strongly inhibited by cellular K (K_c). Inhibition saturated at 10–20 mM $[K]_c$. Surprisingly, the expected inhibition by external K (K_e) was not observed in the absence of K_c. However K_e did inhibit binding if K_c was present. Inhibition was maximal at 200 μM $[K]_e$ and 10 mM $[K]_c$. Na_e inhibited ouabain binding in the absence of K_c or K_e. This is a low affinity Na_e effect, saturating at 10–20 mM. K_c reverses the inhibition by Na_e, such that the binding rate with K_c (10 mM) and Na_e (50 mM) is about the same as that without K or Na. Na_c inhibited ouabain binding, with maximal effect at 5–10 mM. In contrast to K_c, Na_c enhanced inhibition by K_e only slightly. Therefore Na_c and K_c inhibit at separate sites. The results of this study are interpreted in terms of three phosphoenzyme forms: E_1P, E*P, and E_2P. E*P is the form binding ouabain with high affinity (Yoda and Yoda. 1988. *J. Biol. Chem.* 263:10320). K_c promotes E*P → E_2P, thereby inhibiting ouabain binding. K_e binds only to E_2P; therefore K_c is required for inhibition by K_e. Na_e inhibits binding by stabilizing E_1P, while Na_c inhibits by stabilizing E_1. The reversal by K_c of inhibition by Na_e may mean that K_c and Na_e together favor formation of E*P, and that K_c and Na_e bind to the enzyme simultaneously. A role for K_c in interconversion of phosphoenzyme forms is corroborated by this study.

33. Voltage Dependence of the Na^+/K^+ Pump of *Rana* Oocytes MICHAEL M. WU and MORTIMER M. CIVAN, *Departments of Bioengineering, and Physiology and Medicine, University of Pennsylvania, Philadelphia, Pennsylvania*

The current (I_p) through both the forward and backward running Na,K exchange pump in cardiac myocytes and the squid axon has been demonstrated to vary monotonically with membrane potential (V_m) up to (forward running) or down to (backward running) a plateau region. This has been shown to be consistent with a Post-Albers kinetic model in which only the Na^+ translocation is voltage sensitive. With a 3Na:2K stoichiometry, postulating the existence of two negative charges at the ion binding sites of the ATPase suffices to model the voltage sensitivity. The nature of I_p in frog oocytes, on the other hand, has not been as clear. Measured as glycoside-sensitive current, I_p of *Xenopus* oocytes has been reported to depend directly on V_m, peak at ≈20 mV, but to decline thereafter. If correct, this suggests that the K^+ translocation is also voltage sensitive. We have previously shown, however, that the accumulation of K^+ (and possibly other ions) during pump blockage in *Rana pipiens* oocytes can affect parallel, nonpump current pathways, leading to over- or underestimates of I_p. To reduce the effect of parallel currents, we added channel blockers (Ba^{++}, Co^{++}, or Cd^{++}) to the experimental solution. Defolliculated oocytes of *R. pipiens*, which were Na^+ preloaded, were voltage clamped (−150 to 50 mV, or −160 to 40 mV) with and without 2 or 5 μM strophanthidin. Currents were measured while the currents were alternately hyper- and depolarized for 500 ms at intervals of 3 s. After prolonged exposure to the channel blockers, the overall membrane conductance and holding current fluctuations decreased, though voltage-activated currents were not completely blocked. I_p obtained during this period was reproducible and reversible. Averaging 16 difference current measurements obtained with four oocytes, I_p depended directly on V_m up to a plateau at −10–40 mV, and reversed at −149 mV. In another set of oocytes, I_p was found to vary with $[Na_o]$, becoming less sensitive to voltage when $[Na_o]$ was decreased. Contrary to what has been

reported in *Xenopus* oocytes, our results suggest that there is no negative conductance region of I_p. In agreement with previous findings, our results demonstrate that I_p is dependent on $[Na_o]$. [Supported in part by NIH research grant DK-40145.]

34. Permeabilization of Synaptic Terminals with α-Toxin GILBERT J. CHIN* and STEVEN S. VOGEL,* *Howard Hughes Medical Institute and Department of Biochemistry and Molecular Biophysics, Columbia University, New York, New York* (Sponsor: Guido Guidotti)

Intact rat brain synaptosomes (isolated synaptic terminals) release neurotransmitter when depolarized in the presence of external Ca^{2+}. The entry of Ca^{2+} can be regulated by G proteins, which mediate the effects of extracellular ligands on voltage-sensitive Ca^{2+} and K^+ ion channels. Using PC12 cells permeabilized with *Staphylococcus aureus* α-toxin, Ahnert-Hilger et al. (1987. *Biochemistry.* 26:7842) have shown that Ca^{2+}-induced exocytosis can be modulated by a pertussis toxin–sensitive G protein. To look at the possible involvement of GTP-binding proteins in later steps of transmitter release from nerve terminals, we have treated synaptosomes with α-toxin, which forms pores in the synaptic plasma membrane. The insertion of these pores collapsed the membrane potential as determined using a fluorescent cyanine dye. Small cytoplasmic molecules, such as $^{86}Rb^+$, passed freely across the permeabilized membrane, while macromolecules, such as lactate dehydrogenase, were retained. In preliminary experiments, we have observed that acetylcholine release from permeabilized synaptosomes can still be triggered by external Ca^{2+}. We plan to use the α-toxin pores to look at the effects of small molecules, such as guanine nucleotides, or transmitter release.

35. Proton Permeability Of Toad Bladder Aggrephore Membranes: Role of The Water Channel In Proton Transport H. W. HARRIS JR.,* D. KIKERI,* A. JANOSHAZI,* A. K. SOLOMON, and M. L. ZEIDEL,* *Harvard Medical School, Boston, Massachusetts*

In toad urinary bladder antidiuretic hormone (ADH) stimulation induces fusion of cytoplasmic vesicles called aggrephores (whose limiting membranes contain water channels) with apical membrane and markedly increases transepithelial water and proton permeabilities. Termination of ADH stimulation in toad urinary bladder retrieves water channels from apical membrane through endocytosis of aggrephore membrane. Since we have shown that the membrane of endocytosed aggrephores has a high water permeability ($>4.5 \times 10^{-2}$ cm/s, *Clin. Res.* 37:581A), we measured the H^+ permeability of aggrephore membrane after fluorescein dextran was sequestered within these vesicles. Intravesicular pH was monitored by changes in fluorescein fluorescence (excitation, 499 nm; emission, 520 nm; 2 nm slits) under conditions where: (*a*) only intravesicular dye was detected, (*b*) fluorescence intensity was directly proportional to intravesicular pH, (*c*) osmolar changes did not affect the signal, and (*d*) omission of ADH stimulation before vesicle preparation produced no signal. Proton permeability (P_{H^+}) was measured by abruptly lowering extravesicular pH from 8.0 to 6.0 using a stopped-flow device. P_{H^+} was 4×10^{-3} cm/s. *p*-Chloromercuribenzenesulfonate (pCMBS), which inhibits water flux through toad bladder ADH-stimulated and erythrocyte water channels, decreased p_{H^+} by 50%. In contrast, *N*-ethylmaleimide, phloretin, ATP, and Na^+ gradients were without effect. H^+ flux was not rectified and could be distinguished from diffusion through the lipid component of the vesicle membranes. Thus protons appear to rapidly traverse the aqueous pore of the ADH water channel. This pCMBS-inhibitable H^+ flux may be useful in characterization and isolation of the ADH water channel.

36. Kinetic Properties of the Human Lymphocyte Na^+/H^+ Exchanger PASQUALE STRAZZULLO* and MITZY CANESSA, *University of Naples, Naples, Italy; and Endocrine Hypertension Division, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts*

Our kinetic studies of the activation by cell pH of red cell (rbc) Na^+/H^+ exchanger (Na EXC) have shown that hypertensive patients exhibit increased V_{max} and low Hill coefficients (n_{app}) when

compared with normotensive subjects. These properties indicated that the antiporter is abnormally regulated in these patients. We report here the kinetic properties of the Na EXC of human lymphocytes which can also be used to probe the gene encoding this transport system. Monocyte-free lymphocytes from healthy volunteers were prepared by centrifugation in Percoll density gradients. Cell pH was measured using the fluorescent probe BCECF-AM and a modification of the procedure developed by Grinstein et al. for rat thymocyte Na EXC. Acid loading to six different cell pHs (pH_i, 7.1–6.0) was achieved using nigericin in Na-free media. Na_o-dependent H^+ efflux was determined at 37°C from the initial rate (10 s) of alkalinization upon addition of 122 mM Na at pH_o 7.4 and the cell buffering power at each pH_i. Na_o-dependent H^+ efflux was sigmoidally activated by cell pH; a Hill plot gave $N_{app} = 2.14 \pm 0.15$ and $pKa = 6.56 \pm 0.03$ ($n = 6$). The measured V_{max} at pH_i 6.1 was 0.51 ± 0.17 mmol/liter cell water × s (X ± SE, $n = 12$) and varied markedly between individuals. External Na^+-activated H^+ efflux (pH_i 6.2) was a saturable function with $K_m = 30 \pm 6$ mM and calculated $V_{max} = 0.73 \pm 0.06$ mmol/liter cell water × s ($n = 3$). Na_o-dependent H^+ efflux was completely inhibited at pH_o 6.4 with K_i 93 nM H_o and by 200 μM amiloride with an $IC_{50} = 17$ μM. These results indicate that the human lymphocyte has a powerful Na EXC with marked interindividual V_{max} differences.

37. Antibodies Against Proteins of the Na/Cl Cotransporter P. B. DUNHAM, B. DYER,* F. JESSEN,* and E. K. HOFFMAN,* *Department of Biology, Syracuse University, Syracuse, New York; and August Krogh Institute, University of Copenhagen, Copenhagen, Denmark*

We have purified proteins from solubilized membranes of Ehrlich ascites cells using a bumetanide-Sepharose affinity column (Feit et al. 1988. *J. Membr. Biol.* 103:135). We proposed that one or more of these proteins is a constituent of the Na/Cl cotransporter. The evidence, the specificity of binding of bumetanide to the cotransporter (Hoffman et al. 1986. *Am. J. Physiol.* 250:C688), does not prove the hypothesis. We now have proof: antibodies raised against the purified proteins inhibit Na/Cl cotransport in Ehrlich cells. Rabbits were immunized with the purified proteins by injection into the popliteal lymph node. This preparation had major protein bands at 90, 80, 40, and 32 kD, similar to those we reported earlier (Feit et al. 1988). In a western blot of these proteins, the antiserum immunodetected the 90 and 80 kD proteins, and also minor proteins of 65 and 50 kD. Na/Cl cotransport in Ehrlich cells is activated by osmotically induced cell shrinkage (Hoffman et al. 1983. *J. Membr. Biol.* 76:269). The first test for effect of the antiserum on the cotransporter was the regulated increase in cell volume after shrinkage, regulation mediated by cotransport. Cell volumes were measured using a Coulter counter after pretreatment with preimmune serum, after pretreatment with antiserum, in control cells, and under these three conditions with 25 μM bumetanide. Control cells shrank from 1,130 to 585 fl (modal cell volume), then regulated their volumes upward to 800 fl in 5 min. The volume increase was unaffected by preimmune serum, and was inhibited 100% by bumetanide. Regulation was also inhibited 100% by antiserum, demonstrating the presence of antibodies which bind to the cotransporter at the external membrane surface and inhibit its function. This was confirmed by measurements of ^{36}Cl influxes during volume regulation, the second measure of cotransport. In preimmune serum, bumetanide inhibited Cl influx ~40%. Antiserum inhibited ~50% of the bumetanide-inhibitable flux. It is not possible to know which of the proteins recognized by antibodies are part of the Na/Cl cotransporter. However, it is possible to conclude with certainty that at least one of the proteins is a part of it.

38. Volume-sensitive K-Cl Cotransport in Inside-Out Vesicles Made from LK Sheep Erythrocyte Membranes G. R. KRACKE* and P. B. DUNHAM, *Department of Anesthesiology, University of Missouri-Columbia, Columbia, Missouri; and Department of Biology, Syracuse University, Syracuse, New York*

Unidirectional K effluxes were measured from inside-out vesicles (IOVs) prepared from LK sheep red cell membranes (Mercer and Dunham. 1981. *J. Gen. Physiol.* 78:547). K effluxes were measured using ^{86}Rb into media containing, in millimolar, 38 K, 77 Na, 0.8 Mg, 1 ATP, 0.02 stro-

phanthidin (permeant Na pump inhibitor), pH 7.4, and either Cl or $MeSO_3$ as the principal anion. Osmolarity was adjusted with sucrose. Total K efflux (in Cl medium) was 25.8 mmol per l vesicle per h in a medium of 295 mosmol/kg. Cl-dependent K efflux, or K-Cl cotransport, was 9.3 mmol/(l × h), measured as the difference between effluxes in Cl- and $MeSO_3$-media. K-Cl cotransport increased 15% to 10.7 mmol/(l × h) in hypotonic medium, 230 mosmol/kg, and decreased to 5.4 mmol/(l × h) in hypertonic medium, 430 mosmol/kg. While K-Cl cotransport in IOVs increased with swelling, it is much less sensitive to swelling than it is in intact cells. Relative vesicle volumes, measured with [^{3}H]water and [^{14}C]sucrose, increased and decreased reversibly with changes in osmolarity to extents close to those predicted. At 295 mosmol/kg, relative vesicle volume was 5.8 μl H_2O/mg protein. K-Cl cotransport was enhanced by Mg and also by ATP, but not by the ATP analogues AMP-PNP and TNP-ATP. Therefore, phosphorylation of something promotes cotransport. Since swelling of both intact cells and IOVs enhances K-Cl cotransport, the signal detected by the "volume sensor" probably a change in membrane tension, is independent of membrane configuration (right-side-out or inside-out). This first demonstration of volume-sensitive K-Cl cotransport in IOVs provides an in vitro model system for the direct investigation of the volume sensor, a mechanism of unknown nature located at the cytoplasmic membrane surface.

39. The Action of Galanin on Electrolyte Transport and Potassium Channels Activity in Rabbit Ileum FADIA R. HOMAIDAN, LINDA M. NOWAK, MARK DONOWITZ, and GEOFFREY W. G. SHARP, *Department of Pharmacology, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York; and Departments of Gastroenterology and Physiology, The Johns Hopkins University, School of Medicine, Baltimore, Maryland*

The action of galanin on electrolyte transport in rabbit ileum was studied. Galanin, which is secreted from subsets of submucosal neurones, decreases short-circuit current (I_{sc}) with an EC_{50} of 10 nM. I_{sc}, representing the sum of all ion movement across the tissue, was measured by the Ussing-chamber voltage-clamp technique. Unidirectional flux studies, using ^{22}Na and ^{36}Cl show that the decrease in I_{sc} is correlated with an increase in Na and Cl absorption. Tetrodotoxin inhibited 50% of the effect of galanin on I_{sc}, indicating that part of the response is neuronal, but that there may be an additional direct effect on enterocytes. Preliminary studies, using whole-cell patch-clamp recording on dissociated villus cells, indicate that galanin does act directly on the enterocytes and affects voltage-activated potassium channels, presumably located on the basolateral membrane of the cells. Galanin increases whole-cell potassium currents at all potentials without any apparent change in the voltage dependence of activation. Thus, it appears that galanin either increases probability of channel opening, the number of open channels, or the mean channel open time, through some mechanism(s) yet to be identified.

40. Chloride Channel–related Proteins from *Necturus* gallbladder: Common Antigenic Determinants from Different Species L.-M. TSAI,* R. J. FALK,* and A. L. FINN, *Departments of Medicine and Physiology, University of North Carolina School of Medicine, Chapel Hill, North Carolina*

Monoclonal antibodies (MAbs) were raised by injecting Balb/c mice with a nitrogen cavitate of theophylline-treated *Necturus* gallbladder (NGB) cells and were tested for their ability to inhibit the cAMP-dependent apical chloride conductance in that tissue. Five of these antibodies bound to the apical cells by indirect immunofluorescence microscopy and inhibited the chloride conductance; one bound to subepithelial smooth muscle and had no effect on the conductance. The reactivity of the MAbs was also tested by enzyme-linked immunosorbent assay (ELISA) and then more specifically by polyacrylamide gel electrophoresis with Western blot analysis. This report concerns one of these antibodies, which inhibits over 80% of the conductance in NGB. All studies were carried out in tandem with the smooth-muscle MAb as a control; in each case the latter had no effect on any chloride conductance tested. The MAb that inhibited the NGB channel partly blocked the chloride channel in the apical membrane in the *Necturus* small intestine (NI), and completely blocked both

the cAMP-dependent chloride conductance in toad gallbladder (TGB), and the prostaglandin-stimulated chloride conductance in rat colon. By ELISA, this antibody was shown to cross-react with homogenates from these same cell sources as well as from human lymphocytes (HL), which also have a chloride conductance (Grinstein et al. 1982. *J. Gen. Physiol.* 80:801). Western blot analysis demonstrated that it recognized proteins of 219 kD in NGB, TGB, and RC, of 240 and 200 kD in NI, and of 250 and 219 kD in HL, suggesting that these proteins share common or related epitopes with chloride channel–related molecules. [Supported by NIH grants DK-25483 and DK-40208.]

41. Volume-sensitive K-Cl Cotransport: Metabolic Dependence and Inhibition by Vanadate and Fluoride SUSAN T. COKER* and W. CHARLES O'NEILL,* *Departments of Medicine and Physiology, Emory University School of Medicine, Atlanta, Georgia* (Sponsor: O. Froehlich)

Swelling of human red cells activates a volume-sensitive (VS) K-Cl cotransport that mediates regulatory volume decrease in a small fraction of these cells. To determine what role ATP has in this process, K influx was measured in isotonic and hypotonic medium after depletion of cellular ATP, and after exposure of cells to vanadate or fluoride. Preincubation of red cells with 3-deoxyglucose resulted in an inhibition of VS K-Cl cotransport, that paralleled the decline in cellular ATP, without any inhibition of Cl-dependent K influx in isotonic medium. Subsequent incubation with phosphate, glucose, and adenosine fully repleted cellular ATP, but not GTP, and partially restored VS cotransport. Greater restoration of VS cotransport was achieved by incubation with guanosine, which repleted both ATP and GTP. Results identical to ATP depletion were obtained by treating cells with vanadate or fluoride. Inhibition by vanadate required a 60-min incubation with 200 μM vanadate and was partially blocked by inhibiting vanadate uptake with diisothiocyanostilbenedisulfonate (DIDS), indicating that vanadate was acting intracellularly. Inhibition by fluoride was complete at 20 mM and did not require preincubation. The effect of fluoride did not depend on the chloride concentration and it was partially prevented by the addition of deferoxamine to chelate Al, suggesting that the inhibition of VS cotransport was due to AlF_4^-. Neither vanadate nor fluoride inhibited Cl-dependent K influx in isotonic medium, but both agents substantially inhibited ouabain-sensitive K influx. These results demonstrate that depletion of ATP, or treatment with vanadate or fluoride specifically inhibit VS K-Cl cotransport without inhibiting Na-K-2Cl cotransport. This indicates that activation of volume-sensitive K-Cl cotransport involves a phosphohydrolase or phosphotransferase reaction that requires ATP and possibly GTP, and suggests that signalling via protein phosphorylation is occurring. [Supported by NIH K08DK-01643 and the National Kidney Foundation of Georgia.]

42. Membrane Lipid Fluidity Influences the Cl^- Permeability in Exocrine Secretory Granules KENNETH GASSER,* ADAM GOLDSMITH,* and ULRICH HOPFER, *Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio*

Zymogen granule membranes contain Cl^- transporters that become important for primary fluid production after fusion with the apical plasma membrane of acinar cells. To examine the importance of membrane fluidity for regulating Cl^- permeability, we have modulated the fluidity of isolated zymogen granule membranes. Cl^- conductance and Cl^-/HCO_3 exchanges were found to be highly sensitive to benzyl alcohol and octanol induced fluidity changes. These agents increase the transport rate up to 10-fold while maintaining transporter characteristics, such as anion selectivity, SITS inhibition, and stimulation and inhibition by nucleotides. Decreasing the temperature from 37°C to 17°C reverses the benzyl alcohol–induced increase in transport. Measurements of the steady-state fluorescent anisotropy of diphenylhexatriene (DPH) probes show that resting secretory granules from a variety of sources possess a much lower fluidity than the plasma membranes (15% change in membrane anisotropy). The membrane fluidity of different granule preparations measured with trimethyl ammonium-DPH, but not with propionic acid-DPH, exhibits a strong correlation to the Cl^- conductance of these granules. In addition, benzyl alcohol, octanol, and temperature produced changes in lipid fluidity that correlate with the changes in granule Cl^- conductance as well as Cl^-/HCO_3 exchange. [Supported by Cystic Fibrosis Foundation grant Z0298.]

43. Protein Kinase C Modulation of Airway Epithelial NaCl(K) Cotransport CAROLE M. LIEDTKE and GARY WASCOVICH, *Department of Physiology, Case Western Reserve University, Cleveland, Ohio*

In previous work with isolated tracheal (TEC) and nasal (NEC) epithelial cells, we demonstrated Ca-dependent, α-adrenergic activation of loop diuretic-sensitive NaCl(K) cotransport (Liedtke. 1989. *Am. J. Physiol.* In press; Liedtke. 1989. *FASEB. J.* 3:859). We continue these studies by exploring a role for Ca- and diacylglycerol-dependent protein kinase C (PKC) in modulating loop diuretic-sensitive Cl transport in dispersed human and rabbit TEC and human NEC. Cl transport was measured as ion efflux from cells preloaded with radiotracer and is expressed as the initial rate of Cl efflux (nmol Cl/10^6 cells/min). First, inhibition of PKC in rabbit TEC with stausporine reduced the basal rate of Cl transport by 24.5% from 9.8 ± 0.6 (n = 8) to 7.4 ± 2.0 (n = 3) and blunted epinephrine (EPI)-stimulated Cl transport by 62.7% from 17.7 ± 1.3 (n = 4) to 11.1 ± 1.9 (n = 3). Stausporine also blunted EPI-induced Cl fluxes in non-CF TEC. Second, the PKC activator, phorbol 12-myristate 13-acetate (PMA), increased the baseline rate of Cl transport in rabbit TEC by 45.9% to 14.3 ± 1.5 (n = 6) of which 26.6% was sensitive to BMT. In non–cystic fibrosis (CF) TEC, PMA caused a 94.8% increase in basal Cl transport from 9.7 ± 2.2 (n = 4) to 18.9 ± 1.6 (n = 4) that was not further increased by the addition of EPI. In CF cells, however, PMA caused only a 28.0% increase in basal Cl transport from 12.0 ± 0.9 (n = 6) to 15.4 ± 0.4 (n = 3). We conclude from these results that PKC plays a role in the regulation of basal Cl transport when physiological Ca^{2+} levels are low, and in the activation of NaCl(K) cotransport, during hormone stimulation, when Ca^{2+} levels are elevated. [Supported by the NIH (DK-27651) and National Cystic Fibrosis Foundation.]

44. Ionic Current and Unidirectional Potassium Flux in the Resting Membrane of Squid Axons D. C. CHANG and J. R. HUNT,* *Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas*

Understanding the resting potential of the nerve cell is a classical problem which has not been satisfactorily resolved. The traditional model of the membrane potential, the Goldman-Hodgkin-Katz theory, assumed that the resting potential is a simple diffusion potential involving mainly K^+ and Na^+ ions. Very little was known about the molecular properties of the pathways that control the resting currents. In an attempt to gain a better understanding of ion transport properties of the resting membrane, we used the internally perfused squid giant axon as a model to study the ionic pathways at the resting state. The techniques of voltage-clamp and unidirectional ion flux were used in this study. The basic approaches of this study are: (*a*) to correlate the changes of resting current with isotope-labeled K flux, (*b*) to determine the effects of voltage on the resting current and the ^{42}K efflux, and (*c*) to determine the effects of K concentration on the membrane current and ^{42}K efflux at the resting state. The preliminary results of our study indicate that the resting conductance is slightly voltage dependent and behaves significantly different from the delayed rectifier K channel. The ^{42}K efflux also varies with potential near the resting potential, but in a manner much less steep than previously suggested. Furthermore, ^{42}K efflux was found to be stimulated by an increase of external K concentration, rather than inhibited by it. These findings suggest that the resting potassium pathway may be more complicated than a simple mechanical pore. [Supported in part by NIH grant NS-25803-01.]

45. In Vitro Study of the Interactions of Nitrosamine Ligands with the Nicotinic Acetylcholine Receptor from Rectus Abdominis Muscle Preparations of *Xenopus*. C. G. WHITELEY,* *Department of Biochemistry, Rhodes University, Grahamstown, South Africa*

In vitro isolated rectus abdominis muscle preparations show that diphenylnitrosamine (EC_{50} = 0.79 mM) is a more potent agonist to the nicotinic receptor than other aliphatic nitrosamines (EC_{50} = 56–77 mM). Low concentrations of the nicotonic antagonist (*d*-tubocuraine) block the response by aliphatic nitrosamines while much higher concentrations are required against diphenylnitrosamine. Comparisons are made with the natural agaonist, acetylcholine. A twofold

increase in contraction is observed when the muscle is pretreated with nitrosamine before acetylcholine exposure. The response of the muscle contraction with the same nitrosamine after acetylcholine exposure is only 1.5×. The concentration, however, of diphenylnitrosamine is 20 times less than that for dimethylnitrosamine.

46. An Experimental Procedure for Obtaining Aequorin-loaded Isolated Mammalian Cardiac Myocytes ARTHUR J. MEUSE,* CYNTHIA L. PERREAULT,* WILLIAM GROSSMAN,* and JAMES P. MORGAN, *Department of Medicine, Harvard-Thorndike Laboratory and Beth Israel Hospital, Boston, Massachusetts*

The naturally occurring bioluminescent Ca^{2+} indicator aequorin has been successfully used in the detection and quantification of intracellular calcium transients in whole heart and isolated muscle preparations. Aequorin emits light when it combines with Ca^{2+}; light emission is an index of $[Ca^{2+}]_i$. Recently we have developed a chemical loading and enzymatic isolation protocol that allows us to obtain large numbers of aequorin-loaded cardiac myocytes. These loaded, isolated cells maintain morphological and physiological characteristics similar to those seen in unloaded single-cell preparations. Biopsy specimens from the left ventricular free wall of ferret ($n = 8$) were loaded with aequorin using a macroinjection technique described previously (Kihara and Morgan. 1988. *J. Gen. Physiol.* 92:47*a*). The specimens were then dissociated enzymatically with collagenase. Fields of 50–200 cells were electrically stimulated to induce contraction. Video recordings were obtained for subsequent analysis of shortening as an index of contractility, and light emission during twitches of the same cells was recorded with a photomultiplier. The light signals obtained could be manipulated by pharmacological intervention and/or increasing extracellular calcium levels. The configuration and time course of light at 30°C were similar to those recorded with aequorin from intact muscle strips and whole hearts. This protocol is effective in obtaining physiologically active, aequorin-loaded, isolated cardiac myocytes, which are an excellent modality for studying various types of cardiovascular dysfunction.

47. Structure of the Rat Gene Encoding the Dopamine D2 Receptor M. A. MARCHIONNI,* D. K. GRANDY,* M. ALFANO,* J. R. BUNZOW,* and O. CIVELLI,*. *Department of Molecular Biology, Cambridge Neuroscience Research, Cambridge, Massachusetts; and Vollum Institute, Oregon Health Science University, Portland, Oregon* (Sponsor: R. N. McBurney)

A rat gene encoding the dopamine D2 receptor has been cloned, and we have characterized its structure by DNA sequencing and physical mapping. Unlike most members of the family of receptors that couple to G proteins, the coding sequences in the D2 gene are broken up by five introns. We have compared the sequences from several members of this gene family and have produced an alignment and an evolutionary tree. Placement of introns in the rat D2 dopamine receptor gene, when compared with those found in bovine rhodopsin, provide some interesting insights into the evolution of this family of receptors.

48. Bovine Opsin Expressed in Chinese Hamster Ovary Cells Selectively Regulates Adenylyl Cyclase Activity in Response to Light ELLEN R. WEISS,* ROBIN HELLER-HARRISON,* EMILIO DIEZ,* and GARY L. JOHNSON,* *National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado* (Sponsor: Douglas C. Eaton)

The cDNA for bovine opsin, the light-sensitive receptor of the mammalian retinal rod cell, was stably expressed in Chinese hamster ovary (CHO) cells. Membranes derived from individual clones were incubated with 11-*cis* retinal to regenerate the rhodopsin molecule and reconstituted with G_t, the rod cell G protein isolated from bovine rods. The results of GTPγS binding studies demonstrated that rhodopsin expressed in CHO cells is able to couple to G_t, its natural substrate in rod cells, despite an altered molecular mobility of the protein (compared with native opsin) on poly-

acrylamide gels due to heterogeneous glycosylation. In addition, cells incubated with 11-*cis* retinal blocked the intracellular accumulation of cAMP in response to light. This effect was attenuated by preincubation of the cells with pertussis toxin which suggests that bovine rhodopsin expressed in these cells couples to an endogenous G_i-like protein. However, retinal-regenerated rhodopsin had no significant effect on the stimulation of phosphatidylinositol (PI) hydrolysis by thrombin. Since CHO cells have been reported to possess both pertussis-sensitive and pertussis-insensitive pathways for regulating PI hydrolysis (Ashkenazi et al. 1989. *Cell.* 56:487), these data demonstrate the selective interaction of rhodopsin with endogenous G proteins. The rhodopsin polypeptide is the smallest of the G protein-coupled receptors for which sequence data is available and its loop structures are much smaller than those of other receptors. Therefore these structures in opsin represent minimal sequences responsible for the interaction of the receptor with G proteins. The domains of opsin responsible for regulation and selective interaction with G proteins are presently being investigated using site-directed mutagenesis. [Supported by NIH grant GM-30324; E. R. Weiss is supported by NRSA GM-11477.]

49. Comparative Studies of the Phosphorylation of Muscarinic Cholinergic Receptors by Protein Kinase C and the β-Adrenergic Receptor Kinase RICARDO M. RICHARDSON and M. MARLENE HOSEY, *Department of Biological Chemistry and Structure, University of Health Sciences/The Chicago Medical School, North Chicago, Illinois*

Muscarinic cholinergic receptors (mAChR) in the heart couple to several different G protein-linked signal transduction pathways. Studies of chick heart muscarinic cholinergic receptors carried out in situ have shown that phosphorylation of the receptors occurs in an agonist-dependent manner. This process has been linked to receptor desensitization. The mechanism(s) by which agonists induce mAChR phosphorylation are not known with certainty. Purified and reconstituted mAChR are phosphorylated in vitro by the β-adrenergic receptor kinase (βARK) in an agonist-dependent manner with marked similarities to that which is observed in intact cells. This suggests that the mechanism of desensitization by phosphorylation of mAChR might follow a pathway similar to that which has been proposed for adrenergic receptors. However, other mechanisms may also regulate mAChR function. In this work, we present evidence that protein kinase C (PKC), purified from chick brain, can also phosphorylate the purified, reconstituted chick heart mAChR. The PKC-mediated phosphorylation of mAChR is not agonist dependent. Phosphorylation of the mAChR with PKC occurs to an extent of ~5 mol P/mol receptor in the presence or absence of a mAChR agonist. Kinetic analysis of the additive phosphorylation of the mAChR by PKC and βARK reveals that phosphorylation by these kinases is partially additive. As mAChR may activate PKC through hydrolysis of polyphosphoinositides, these results suggest that PKC may also participate in the regulation of mAChR function by phosphorylation. [Supported by NIH grant HL-31601.]

50. Anti-Peptide Antibodies Detect Muscarinic Cholinergic Receptor Subtypes in Brain and Heart CHANDRA SHEKHAR K. MAYANIL and M. MARLENE HOSEY, *Department of Biological Chemistry and Structure, Chicago Medical School, North Chicago, Illinois*

It is established that different subtypes of muscarinic cholinergic receptors (mAChR) exist and that they couple to different G protein-linked signal transduction pathways. To obtain specific tools with which to study the properties of the different mAChR subtypes, antisera to a number of synthetic peptides corresponding to sequences of mAChR subtypes M1 and M2 (Peralta et al. 1987. *EMBO J.* 6:3923) have been prepared. Hydrophilic peptides from each subtype were chosen from the large 5-6_i loops (intracellular loop between transmembrane segment 5 and 6) because these loops: (*a*) are distinct in each of the mAChR subtypes, (*b*) may contain determinants for coupling of different mAChR subtypes to different G proteins and effectors, and (*c*) are likely to contain sites for phosphorylation. Anti-M1 antibodies were prepared against HM1 residues 210–223 and 280–293, and anti-M2 antibodies were prepared against HM2 residues 277–290. The antibodies were tested for their reactivities towards mAChR in rat brain synaptosomes which contains ~60% M1 mAChR and lesser amounts of other subtypes, and porcine heart membranes which may contain

only the M2 subtype. The M1 antisera immunoprecipitated 55–60% of the [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding activity solubilized from rat brain synaptosomal membranes, but only ~10% of the mAChR from porcine heart. The anti-M2 antibodies immunoprecipitated 10–15% [^{3}H]QNB binding activity from rat brain synaptosomal membranes, but 60–65% from solubilized porcine heart membranes. The results suggest that the antisera specifically recognize the mAChR subtypes; therefore, these antibodies should be useful tools to study the various properties of mAChR subtypes. [Supported by NIH grant HL-31601.]

51. Identification and Isolation of a Bacterial Exotoxin Inhibitory Protein from the Ovotestis of *Aplysia californica* That Inhibits ADP Ribosylation

MARK R. HELLMICH* and FELIX STRUMWASSER, *Laboratory of Neuroendocrinology, Marine Biological Laboratory, Woods Hole, Massachusetts*

Our interest in elucidating the mechanism by which the neuropeptide egg-laying hormone (ELH) stimulates egg release from the ovotestis of the marine opisthobranch mollusc *Aplysia californica* resulted in the discovery of a potent inhibitor of bacterial exotoxin-catalyzed ADP-ribosylation (ADPR). Two exotoxins, cholera toxin (CTX) and pertussis toxin (PTX), were used initially to identify GTP-binding proteins (G proteins) in various tissues (abdominal and pleural-pedal ganglia, heart, large hermaphroditic duct, and ovotestis) of *A. californica.* All of the tissues studied, with the exception of the ovotestis, exhibited toxin-specific ADPR of G proteins. Mixing the ovotestis tissue with tissues normally displaying CTX- or PTX-specific ADPR resulted in inhibition of the reaction. The inhibitory factor was isolated from the aqueous soluble fraction of the ovotestis in a three step chromatographic procedure, including cation exchange, gel-filtration, and hydroxylapatite columns. Bacterial exotoxin inhibitory protein (BEIP) has a molecular weight of 29 kD and a pI greater than 9. It inhibits CTX- and PTX-catalyzed ADPR of G proteins in various tissues of *A. californica* and rabbit as well as ADPR of frog transducin (collaboration with Dr. Ete Szuts, Marine Biological Laboratory, Woods Hole, MA) and auto-ADPR of CTX-Al and PTX-Sl catalytic subunits. The 50% inhibitory concentration of purified BEIP is in the range of 0.6–1.7 nM. Experiments indicate that it is unlikely that BEIP is a protease, a glycohydrolase (NADase), or a de-ADPR enzyme although the stoichiometry of inhibition by BEIP suggests that it is acting enzymatically. The sequence of the first 30 NH_2-terminal amino acid residues has been determined. Immunohistochemical analysis, of fixed (4% para-formaldehyde) *A. californica* egg-string with BEIP-specific monoclonal antibodies, reveals specific staining associated with the animal's eggs. One possible function for BEIP might be to protect the eggs from attack by exotoxin-secreting bacteria after they have been laid. [Supported by NIH grant NS-21046.]

52. Production and Screening of an Ovotestis cDNA Library for Bacterial Exotoxin Inhibitory Protein Genes

DAVID L. GLICK,* MARK R. HELLMICH,* PAUL TEMPST,* and FELIX STRUMWASSER, *Laboratory of Neuroendocrinology, Marine Biological Laboratory, Woods Hole; and Department of Genetics, Harvard Medical School, Massachusetts*

Bacterial exotoxin inhibitory protein (BEIP) is an inhibitor of cholera and pertussis toxin catalyzed ADP-ribosylation. This 29-kD protein has been purified to homogeneity from the ovotestis of *Aplysia californica* (Hellmich and Strumwasser, companion abstract). In the interest of cloning the gene for BEIP, an ovotestis cDNA library was produced by the random priming of poly-A+ ovotestis mRNA. After second-strand synthesis, Not I, EcoR I adapters were added and the cDNA was size selected on a BioRad A-50 column. Ligation into lamba gt-10 resulted in ~1×10^7 recombinants. About 0.045% of the recombinant plaques hybridized to an *Aplysia* actin probe, indicating that the library is likely to contain most genes expressed in the ovotestis. To produce probes for screening the library the first 30 NH_2-terminal amino acid residues of BEIP was obtained by automatic Edman degradation in two independent runs. In addition, internal sequence was obtained by digesting BEIP with endoproteinase Lys-C, purifying the peptides by HPLC, and determining their sequence. By looking for areas of lowest degeneracy and using Inosine in ambiguous codon posi-

tions, NH_2-terminal and internal oligonucleotide probes were designed and will be used to screen the library. The NH_2-terminal probe is a 20 mer with 32-fold degeneracy and the internal probe is a 23 mer with 64-fold degeneracy. Plaques showing hybridization with both probes will be selected and further characterized. [Supported by NIH grant NS-21046 to F. S.]

53. Calculation and Presentation of Receptor Occupancy Surfaces MARGARET E. KARGACIN,* CHERYL R. SCHEID, and THOMAS W. HONEYMAN,* *Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts*

Pharmacological and genetic studies have defined at least five subtypes of muscarinic receptors. Pharmacological and genetic evidence indicates that many cell types express more than a single receptor subtype. There is also evidence that different receptor subtypes may be discretely coupled to separate biochemical or physiological process by means of G proteins. Gastric smooth muscle contains a mixture of at least two subtypes of muscarinic receptor, M_3 and M_2, defined by the measured binding constants of selective antagonists (Lucchesi et al. 1989. *Nauyn-Schmeidberg's Arch. Pharmacol.* 339:145–151). Further, these receptors can exist as an equilibrium between G protein-coupled receptors with high agonist affinity and free receptors with low agonist affinity. Since one or more of these four species of receptor (M_3 and M_2; coupled or free) may initiate the muscarinic response it is necessary to define conditions whereby each species of receptor may be independently stimulated by agonist. A series of equations was written defining the interaction of agonists and antagonists with each of the four species of receptor. This set of 14 equations was solved for occupancy of each species by agonist and antagonist by matrix inversion. The solutions could be conveniently displayed by plotting, as a 3D surface, the agonist occupancy of each species as a function of varying concentrations of agonist and antagonist. To predict conditions in gastric muscle whereby individual receptor species may be selectively occupied by agonist, surfaces describing the occupancy of each receptor species were compared. This comparison predicts that individual receptor species can be selectively occupied by agonists in a graded manner. Thus the biological consequences of stimulation of each receptor species can be examined independently.

54. Regulation of cAMP Synthesis of $G\alpha_S/G\alpha_i$ Chimeras in Cos-1 and Chinese Hamster Ovary Cells SHOJI OSAWA,* LYNN HEASLEY,* and GARY L. JOHNSON,* *National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado* (Sponsor: Douglas C. Eaton)

G_s and G_i are G proteins that regulate stimulation and inhibition of adenylyl cyclase, respectively. We have constructed a chimeric cDNA referred to as $\alpha_{i(54)/s}$ in which the sequence encoding the amino-terminal 61 amino acids of the alpha subunit of G_s (α_s) was replaced with the first 54 residues from α_{i2}. Transient expression of the $\alpha_{i(54)/s}$ chimera in Cos-1 cells increased cyclic AMP levels two- to threefold in the absence and 18–20-fold in the presence of the phosphodiesterase inhibitor, methyl isobutylxanthine (MIX). Expression of wild type α_s in Cos-1 cells had little effect on basal cyclic AMP levels in the absence of MIX, but increased cyclic AMP levels five to sixfold in the presence of MIX. Stable expression of the chimeric $\alpha_{i(54)/s}$ cDNA in Chinese hamster ovary (CHO) cells constitutively increased cyclic AMP synthesis and cyclic AMP–dependent protein kinase activity. Cyclic AMP levels and cyclic AMP–dependent protein kinase activity were similar to those of control CHO cells in clones expressing wild-type α_s. A second α_i/α_s chimera that contains the NH_2-terminal 60% of α_{i2} and the COOH-terminal 40% of α_s (60/40 α_i/α_s) did not constitutively activate cAMP synthesis but behaved like the wild-type α_s polypeptide in both stable and transient expression assays. A third chimera, $\alpha_{s/i(38)}$, has the last 36 amino acids of the α_{i2} polypeptide substituted for the COOH-terminal 38 amino acids of α_s. Transient expression of $\alpha_{s/i(38)}$ in Cos-1 cells indicated that this chimera was a functional α_s polypeptide. Stable expression of $\alpha_{s/i(38)}$ in CHO cells showed higher levels of cAMP compared with expression of the wild-type α_s subunit, and constitutively activated cAMP-dependent protein kinase. Taken together, our results define the extreme NH_2 terminus of α_s as a regulatory domain controlling basal G_s activity and that the functional α_s domain required for adenylyl cyclase stimulation resides within amino acids 235–356 of the α_s polypeptide. [Supported by NIH grants GM-30325 and DK-3878.]

55. Pertussis Toxin–sensitive G Proteins Are Transported by Fast Axonal Transport Toward Nerve Terminals STEVEN S. VOGEL,* GILBERT J. CHIN,* and THOMAS S. REESE,* *Marine Biological Laboratory, Woods Hole, Massachusetts; Howard Hughes Medical Institute, Columbia University, New York, New York; and National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland* (Sponsor: Guido Guidotti)

Pertussis toxin (PTX)–sensitive G proteins have been found both on plasma membranes and on intracellular membranes in nonneuronal cells. Using PTX-catalyzed [^{32}P]ADP-ribosylation, we find that 43% of the toxin substrate in the squid giant axon is recovered in extruded axoplasm. To determine the direction and mode of transport of cytoplasmic G proteins, we blocked axonal transport by focally cooling a 1-mm segment of axon to 4°C for 2 h while maintaining the rest of the axon at 16°C. Fahim et al. (1985. *J. Neurocytol.* 14:689) have shown that anterograde transport vesicles accumulate on the proximal side of a temperature block and retrograde transport vesicles accumulate on the distal side. In homogenates prepared from 5-mm segments proximal to the block there was twice as much PTX-sensitive G protein as in distal segments (six of seven axons); without focal cooling there was no difference. A similar buildup of G protein was observed using an antibody that recognizes an epitope shared by all G proteins. In the same samples, the overall Coomassie blue staining as well as actin, tubulin, and neurofilament subunits were distributed equally in proximal and distal segments. We calculate that the net rate of G protein transport in the squid giant axon is 44 mm/d at 16°C, consistent with the rate of fast axonal transport. Thus, in axons, PTX-sensitive G proteins are found in the cytoplasmic compartment and are transported on anterograde moving vesicles by fast axonal transport. In preliminary work, we find that the PTX-sensitive G protein in axoplasm migrates as a single electrophoretic component and is converted to a doublet after transit to the axolemma, suggesting a posttranslational modification. [S.S.V. received a 1988 Grass Fellowship.]

56. Characterization of the Human $G_{i2}\alpha$ Subunit Gene Promoter LEE S. WEINSTEIN,* INNA KATZ,* ALLEN M. SPIEGEL,* and ANTHONY D. CARTER,* *Molecular Pathophysiology Branch, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland* (Sponsor: Joshua Zimmerberg)

We have previously cloned the human $G_{i2}\alpha$ subunit gene and reported its promoter to be very GC rich, to contain multiple GC boxes (potential Sp1 factor binding sites) and no TATA box (1988. *FEBS Lett.* 232:333), which is consistent with the features of promoters of other constitutively active genes. Subsequently the promoter region from bases −1,214 to +115 relative to the major transcriptional initiation site was cloned upstream of the coding region for chloramphenicol acetyltransferase (CAT) and 5′ deletion mutants were generated using exonuclease III. In experiments in CU-1 monkey kidney cells measuring transient expression of CAT after transfection by calcium phosphate precipitation, multiple mutants between −1,214/+115 and −103/+115 showed similar activities (~10–15 times a promoterless CAT construct) but a −18/+115 construct had much lower activity. Further deletion mutants between −101 and −18 are presently being analyzed to determine the minimal necessary promotor elements for basal expression. Primer extension of total RNA extracted from CU-1 cells previously transfected with the −101/+115 mutant using a CAT-specific primer confirms that the major transcriptional start site of the $G_{i2}\alpha$ promoter/CAT construct is identical to the site in the endogenous gene. 3′ deletion mutants beyond this site have markedly decreased CAT activity, further supporting the location of the start site. Preliminary experiments indicate that the CAT expression of the −1,214/+115 construct is inducible by 9 h of treatment with 1 mM 8-bromocyclic AMP in JEG3 choriocarcinoma cells, consistent with a potential AP1 transacting factor binding site in the upstream promoter. Further studies with these constructs are necessary to dissect important promoter elements regulating the tissue-specific and inducible expression of $G_{i2}\alpha$.

57. Stable Expression of Heavy Metal–inducible G Protein α_i2 and α_i3 Subunit Genes in LLC-PK_1 Cells L. ERCOLANI,* J. STOW,* J. BOYLE,* and D. A. AUSIELLO, *Renal Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts*

Porcine-derived kidney LLC-PK_1 cells have a basolateral membrane adenylyl cyclase and an apical membrane amiloride-sensitive Na^+ channel which we have shown can be regulated by α_i subunits of G proteins. Using specific α_i cDNA probes, this cell type was demonstrated to have α_i2 and α_i3 mRNAs. An attempt to develop mutants overexpressing or lacking α_i subunits was undertaken by recombinant DNA techniques to study the effects of altered α_i subunit expression. A eukaryotic plasmid expression vector (PM-XX) was created with a Zn^{2+} and Cd^{2+} regulated promoter from the mouse metallothionein I-gene. cDNAs encoding the translated regions of the α_i2 or α_i3 gene were then inserted in a sense (α_i2+), (α_i3+) or anti-sense orientation (α_i2-), (α_i3-) in a Eco RI restriction site to be driven by the inducible metal promoter-enhancer of this construct. A second plasmid pMH140 carrying a neomycin resistance gene was cotransfected with either PM-XX or PM-XX containing α_i cDNAs by calcium phosphate precipitation followed by culture and selection with the antibiotic G418. After multiple cell cycles, LLC-PK_1 cells resistant to G418 were obtained. Northern blot analysis of total RNA from individual stably transfected cell lines revealed new, but faint, 3.2-kb α_i2 mRNAs in α_i2+ or α_i2- cells and 4.0-kb α_i3 mRNAs in α_i3+ or α_i3- cells. The addition of 2 μM $CdCl_2$ to these cell lines increased, respectively, the content of the 3.2-kb α_i2 and the 4.0-kb α_i3 transcripts 10-fold, exceeding in amount their nonrecombinant mRNAs for α_i2 and α_i3. Induction with $CdCl_2$ was seen by 30 min of culture. After 12 h exposure of these cells to 2 μM $CdCl_2$, antibodies (gift of Dr. Allen M. Spiegel) specific for α_i2 subunits revealed increases only in the basolateral membrane staining of α_i2+ cells but not α_i3+ or α_i3- cells, and decreases in α_i2- cells. By contrast, antibodies specific for α_i3 subunits revealed increases only in the apical membrane staining of α_i3+ cells but not α_i2+ or α_i2- cells, and decreases in α_i3- cells. These data demonstrate (*a*) the stable transfection and regulated expression of two α_i subunit genes in a eukaryotic cell line, and (*b*) for the first time, the geographic distribution of different α_i subunits.

58. The G Protein Subunit α_{il}, but Not α_s, Is Myristylated in COS Cells Transfected with the Genes for Those Subunits TERESA L. Z. JONES,* JOHN J. MERENDINO,* WILLIAM F. SIMONDS,* and ALLEN M. SPIEGEL* *Molecular Pathophysiology Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland* (Sponsor: Joshua Zimmerberg)

The membrane attachment of G protein α subunits may occur through myristylation of their amino termini in addition to association with the β-γ subunits. Myristylation could explain the continued membrane attachment of the α subunits after activation and possible dissociation from the β-γ subunits. Earlier studies by Buss et al. (1987. *Proc. Natl. Acad. Sci.* 84:7493) showed that α_o and α_i, but not α_s or α_{td}, are myristylated. Since α_s is attached to the membrane like the other α subunits, we investigated further whether the 52-kD form of α_s is myristylated in COS cells expressing an increased amount of that subunit. COS cells were transfected with a pCD vector containing either the cDNA for α_s or α_{il}. The proteins were radiolabeled in vivo with [^{35}S]methionine or [^{3}H]myristic acid, fractionated by high speed centrifugation, and immunoprecipitated with affinity-purified antibodies RM and AS, specific for α_s and $\alpha_{il,2}$, respectively. The membrane fraction of the [^{35}S]methionine-labeled cells showed a marked increase in α_s (52-kD form) and in α_{il} compared with the relatively small amounts of the native α_s and α_i found in the nontransfected COS cells. The membrane fraction from the [^{3}H]myristic acid–labeled cells showed incorporation of that label in the transfected α_{il} protein and the native α_i proteins but not the native or transfected α_s proteins. To further evaluate the role of myristylation in membrane attachment the techniques of site-directed mutagenesis were used to cause a substitution of the amino-terminal glycine for an alanine in the α_{il} subunit. Future studies with this mutant will assess whether this substitution prevents myristylation and changes the membrane attachment of the α_{il} subunit.

59. Characterization of $p25^{rab3A}$, a Member of the *ras* Gene Superfamily ETHAN S. BURSTEIN, ADRIANA MOSCUCCI, ALAN WOLFMAN, and IAN G. MACARA, *Environmental Health Sciences Center, University of Rochester, Rochester, New York* (Sponsor: Shey-Shing Sheu)

We detected a 25-kD guanine nucleotide-binding protein by immunoblotting with an anti-peptide antiserum developed against a sequence near the COOH terminus of $p25^{rab3A}$. This protein was observed in brain membranes and cytosol but not in other tissues, although an intense band of 23 kD was observed in skeletal muscle. Immunostaining of coronal sections of the rat brain diencephalon revealed that $p25^{rab3A}$ is expressed exclusively in neurons, particularly in the supraoptic and arcuate nuclei and the periventricular region of the hypothalamus. On a MonoQ high-resolution ion exchange column, this protein coeluted with a 25-kD GTP-binding protein at a salt concentration similar to that known to elute purified $p25^{rab3A}$. This protein possesses intrinsic GTPase activity with a rate constant of 0.026/min. In the presence of rat brain cytosol, the GTPase activity was increased twofold. GDP binding was not inhibited by *N*-ethylmaleimide. The dissociation constant for GDP was 100-fold smaller in the presence of Mg^{2+} than in the presence of excess EDTA. Current work is aimed at purifying $p25^{rab3A}$ to homogeneity so that the kinetics of nucleotide and Mg^{2+} binding and the regulation of GTPase activity of cytosolic factor(s) may be further investigated.

60. The Role of Intracellular Messengers in the Regulation of Muscarinic ACh Receptors in Rat Cerebral Cortex Slices C. SHAW,* F. VAN HUIZEN,* and M. CYNADER,* *Department of Ophthalmology, University of British Columbia, Vancouver, British Columbia, Canada*

Muscarinic ACh receptors (mAChRs) were characterized in rat cerebral cortex slices (Van Huizen et al. 1989. *Mol. Brain Res.* 5:59). Down-regulation of mAChRs was observed by increasing neural activity with veratridine or by activating the receptors with carbachol (Shaw et al. 1989. *Mol. Brain Res.* 5:71). The effect can be blocked by the K^+ channel blockers TEA and apamin. To investigate the role of intracellular messengers in receptor down-regulation, slices were incubated for 4 h at 37°C with a wide variety of drugs acting on second messenger systems before being incubated for 2 h at 4°C with [^{3}H](*N*-methylscopolamine) to label surface mAChRs. Forskolin and choleratoxin (activators of adenylate cyclase), NEM (inactivator of G_i proteins), and arachidonic acid (activator of pertussis toxin–sensitive G proteins) had no effect on mAChR number. GDPβs (a GDP analogue) also had no effect. Of the GTP analogues Gpp(NH)p, and GTPγs, the latter had a significant effect on mAChR number (−9.0%), which could only be partially blocked by simultaneously adding TEA (−6.3%). Since activating or blocking the adenylate cyclase second messenger system had no effect on mAChR number, we believe that mAChR down-regulation is modulated by the IP_3 system, especially since stimulating PKC with phorbol esters causes down-regulation of mAChRs (Jia et al. 1989. *Mol. Brain Res.* In press).

61. Second Messengers in Vasoactive Intestinal Polypeptide–mediated Relaxation of the Cat Lower Esophageal Sphincter S. SZEWCZAK, J. BEHAR, C. HILLEMEIER, J. M. MARSHALL, B. Y. RHIM, and P. BIANCANI, *Rhode Island Hospital and Brown University, Providence, Rhode Island; and University of Michigan, Ann Arbor, Michigan*

Vasoactive intestinal polypeptide (VIP) may be a neurotransmitter responsible for relaxation of some gastrointestinal sphincters. It is known to activate adenylate cyclase causing formation of cAMP. We now show that it also reduces inositol triphosphate (IP_3) levels in the lower esophageal sphincter (LES). Contraction of LES depends on intracellular calcium release through in IP_3-mediated pathway, since: (*a*) incubation in Ca^{++} free physiologic solution (PS) abolished contraction of esophageal cells isolated by enzymatic digestion, but had no effect on contraction of LES muscle cells. (*b*) Incubation in Sr^{++}, which inhibits release of intracellular calcium, abolished contraction in LES cells, but not in the esophagus. (*c*) After permeabilization with saponin and incubation in Ca^{++}-free PS, LES, but not esophageal cells, contracted in response to IP_3. The contraction was

antagonized by the naphthalanesulphonamide W7, which in normal Ca^{++} PS blocked LES, but not esophageal contraction in response to ACh. Thus LES contraction in response to ACh depends on IP_3-mediated release of intracellular Ca^{++}. IP_3 resting levels, measured by ion exchange chromatography, were at least three times greater in LES than in the esophagus. Upon stimulation with maximal ACh, inositol phosphates cycled rapidly, with IP_3 peaking within 2–5 s after addition of the drug. Conversely, upon stimulation with VIP, inositol phosphates decreased rapidly in the LES, while tissue levels of cAMP increased. These changes occurred within 5 s and preceded the onset of LES depend on release of intracellular calcium through an IP_3-dependent pathway, and that VIP-induced relaxation may be associated both with an increase in cAMP and a concomitant decrease in IP_3.

62. Evidence for the Involvement of G Proteins in Progesterone-induced Maturation of *Xenopus laevis* Oocytes R. J. CORK,* M. TAYLOR,* and K. R. ROBINSON, *Department of Biological Sciences, Purdue University, West Lafayette, Indiana*

GTP-γ-S inhibits progesterone-induced maturation of *Xenopus laevis* oocytes and induces a rise in cAMP levels. Maturation induced by maturation-promoting factor is unaffected by GTP-γ-S and although it also prevents the progesterone-induced increases in protein synthesis and protein phosphorylation it has no effect on the basal rates of either. GDP-β-S effects are not easily interpreted but it may cause partial or aberrant maturation in some oocytes. These results suggest that a G protein is involved in the second messenger pathway affected by progesterone but the identity of this G protein and mode of action of progesterone remain unclear. [Supported by NSF grant PCM-8315719.]

63. Effects of 4β-Phorbol-12-Myristate 13-Acetate (PMA) on the Hepatic α_1-Adrenergic Signal Transduction System JOHN F. BEELER* and RONALD H. COOPER,* *Department of Pharmacology, University of South Carolina School of Medicine, Columbia, South Carolina*

As part of a continuing investigation into mechanisms responsible for PMA-induced inhibition of the hepatic α_1 signal transduction process (Cooper et al. 1985. *J. Biol. Chem.* 260:3281) effects of PMA on the α_1 receptor have been examined. Plasma membranes were prepared from control and PMA-treated rat hepatocytes by Percoll density gradient centrifugation, and α_1 receptors were quantitated by [^{3}H]prazosin binding. In membranes prepared from vehicle (dimethylformamide) and PMA-treated hepatocytes, the number of [^{3}H]prazosin binding sites was 802 ± 128 fmol/mg protein and 481 ± 14 fmol/mg protein (n = 3), respectively, while the affinity for [^{3}H]prazosin in vehicle and PMA-treated membranes was 0.121 ± 0.034 nM and 0.095 ± 0.024 nM (n = 3), respectively. The 40% decrease in α_1 receptors was evident 15 min after PMA exposure (1,000 ng/ml) and half-maximal loss of α_1 receptors occurred at ~15 ng/ml. Incubation of membranes prepared from cells exposed to PMA (1,000 ng/ml) for 30 min) with alkaline phosphatase did not reverse PMA-induced α_1 receptor down-regulation. Treatment of hepatocytes with primaquine (300 μM for 30 min) decreased α_1 receptors in plasma membranes by 34% (n = 3), suggesting that the α_1 receptor undergoes recycling. PMA could decrease plasma membrane–associated α_1 receptors either by facilitating internalization or by inhibiting return to the membrane. However, the decrease in membrane-associated α_1 receptors observed in this study cannot account for the acute inhibitory effects of PMA on α_1 -mediated responses, suggesting that PMA may exert multiple inhibitory effects on the hepatic α_1-adrenergic signal transduction system. [Supported by NIH grant DK-33795.]

64. Comparison of Roles of Protein Kinase C, Inositol Trisphosphate, and Ca^{2+} in Cholinergic and α_1-Adrenergic Agonist Stimulation of Lacrimal Gland Protein Secretion ROBIN R. HODGES,* DEANNA M. DICKER,* SAMUEL C. YIU,* and DARLENE A. DARTT, *Immunology Unit, Eye Research Institute, and Ophthalmology Department, Harvard Medical School, Boston, Massachusetts*

Lacrimal gland protein secretion is additive upon the simultaneous addition of the cholinergic agonist carbachol (C) and the α_1-adrenergic agonist phenylephrine (P). To determine the signal transduction pathways used by these agonists, rat exorbital lacrimal glands were analyzed for protein kinase C (PKC) activity, inositol trisphosphate (IP_3) production, and cytosolic Ca^{2+} concentration. To measure PKC activity, acini or pieces were incubated for 20–30 min with either no additions, addition of C, or addition of P, and homogenized. The cytosol (centrifuged 100,000 *g* for 60 min) was incubated with [^{32}P]ATP (5 μM), histone H-1 (0.4 mg/ml), phosphatidylserine (0.3 mg/ml), Ca^{2+} (100 μM), and 4-β-phorbol 12,13-dibutyrate (3 μM), and then ^{32}P incorporation was measured. To measure IP_3 production, acini were preincubated with 100 μCi/ml myo-[^{3}H]inositol and stimulated with either no additions, addition of C, or addition of P. IP_3 was determined by anion exchange chromatography. To measure Ca^{2+}, acini were incubated for 60 min in quin-2 ester. Ca^{2+} was determined after addition of either C or P. Basal PKC activity was 109.6 ± 20.1 pmol ^{32}P incorporation/mg protein (n = 3). A maximum translocation of PKC from the cytosol occurred with 10^{-4} M P, which decreased PKC activity by 84%. C (10^{-5} M) decreased PKC activity by 95% from a basal value of 317.5 ± 91.3 (n = 3). A 1-min incubation with C (10^{-5} M) significantly increased IP_3 production (IP_3/inositol ratio) twofold from 0.0054 ± 0.0008 (n = 4); a 20-min incubation increased it fivefold. P (10^{-2}–10^{-5} M) did not change the IP_3/I ratio. C (10^{-4} M) significantly increased Ca^{2+} from 158 ± 20 to 611 ± 46 nM (n = 10). With P (10^{-4} M), the Ca^{2+} was unchanged at 134 ± 46 nM (n = 6). We concluded that cholinergic agonists use IP_3, Ca^{2+}, and PKC, and α_1-adrenergic agonists use only PKC, thus accounting for the additivity of protein secretion. [Supported by NIH grant EY-06177, Fight For Sight, and Lions Clubs of Massachusetts.]

65. Immortalized Cystic Fibrosis Airway Cells Retain a Defective cAMP-stimulated Cl^- Channel DANIEL WOLFF,* MITZY CANESSA, JOSE ROMERO,* and LESLIE KRUEGER,* *Endocrine-Hypertension Division, Harvard Medical School, Boston, Massachusetts; and Division of Genetics, Hahnemann University, Philadelphia, Pennsylvania*

Airway epithelial cells from normal cystic fibrosis (CF) subjects have limited in vitro survival for studying their biochemical and transport abnormalities. Immortal airway cells (IAC) provide a homogenous cell population for genetic and biochemical studies to probe the mechanisms of the defective secretory response of the Cl^- channel. IAC from normal and from CF nasal polyp cells transfected with an AD5-SV40 *ori*$^-$ hybrid virus were used to study Cl^- permeability. $^{36}Cl^-$ efflux was measured from both cell types in passages 30–110 determining the radioactivity remaining in the cells as a function of time; a nonlinear regression computer algorithm was used to fit the experimental points to a two-compartment model with fast (k_1) and slow (k_2) rate constants. Two electroneutral pathways, the bumetanide-sensitive Na-K-Cl cotransport (k_2, 0.012 min^{-1}) and a DIDS-sensitive anion exchanger (k_2, 0.100 min^{-1}) accounted for 10% and 77% of the total Cl^- efflux, respectively. Net Cl^- efflux into gluconate media (conductive pathway) was 15% of the flux (k_2, 0.030 min^{-1}). These three pathways have a similar k_2 in the control and CF cell lines. Incubation of normal IAC with db_2-cAMP (1 mM) stimulated conductive Cl^- efflux from 0.029 ± 0.002 to 0.041 ± 0.003 (X ± SD, n = 10). In CF cultures, the basal and db_2-cAMP–exposed levels of conductive Cl^- efflux were similar (0.029 ± 0.003 and 0.031 ± 0.003, n = 6). Additionally, pretreatment with cholera toxin (100 ng/ml) stimulated conductive Cl^- efflux from 0.025 ± 0.001 to 0.041 ± 0.001 (n = 4) in normal IAC but not in CF cells (0.026 ± 0.002 to 0.028 ± 0.003, n = 4). These results are in agreement with previous electrophysiological data which indicate that there is a regulatory lesion in the cAMP-dependent activation of Cl^- channels in CF cells. Additionally, we confirm the retention of the lesion in the cAMP-dependent activation of Cl^- channels in immortalized CF cells using a radiotracer assay.

66. Participation of a G Protein Modulation System on the Assembly and Sealing of Tight Junctions BALDA MARIA SUSANA,* GONZALEZ-MARISCAL LORENZA,* CONTRERAS RUBEN GERARDO,* and CEREIJIDO MARCELINO, *Department of Physiology, Center of Research and Advanced Studies, Mexico City, Mexico*

MDCK cells plated at confluence form monolayers that establish tight junctions (TJ) and develop transepithelial electrical resistance (TER), through a process that requires cell-to-cell contact and Ca^{++}. Yet if shortly after plating, monolayers are transferred to media without Ca^{++}, they do synthesize junctional components, but they are retained in an intracellular compartment, and TER remains negligible. Addition of Ca^{++} to these monolayers ("Ca-switch") provokes an exocytotic fusion that increases surface membrane and rapidly develops TER (4–6 h instead of 12–15). Pertussis toxin (PTX) added along with Ca^{++} increased TER during the Ca^{++}-switch, while aluminum fluoride inhibited this process, suggesting the participation of an inhibitory G protein (G_i). Such G_i does not seem to modulate TJ formation through adenylate cyclase, for addition of IBMX (3-isobutyl-1-methylxanthine), dB-cAMP, or forskolin reduced the values of TER. Instead activation of phospholipase C by TRH increased TER, and the inhibitor neomycin blocked TER development. diC8, an activator of protein kinase C (PKC) increased TER, while the inhibitors sphingosine, polymyxin B, and H7 abolished TER development. Chlorpromazine, trifluoperazine, and calmidazoline inhibited TER, suggesting a role for calmodulin in junction formation. These results propose that during a Ca switch, TJ formation is modulated by a network of reactions where a G_i protein, phospholipase C, PKC, and calmodulin are involved. [Supported by research grants from COSBEL S.A. de C.V. and the National Research Council of Mexico (CONACyT).]

67. G_i Protein Gates a Cation Channel in Renal Inner Medullary Collecting Duct Cells DOUGLAS B. LIGHT, DENNIS A. AUSIELLO, and BRUCE A. STANTON, *Department of Physiology, Dartmouth Medical School, Hanover, New Hampshire; and Renal Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts*

We examined whether G proteins regulate cation channels (28 pS; $P_{Na}:P_K = 1:1$) in the apical membrane of the renal inner medullary collecting duct (IMCD) and whether they modulate Na^+ absorption. Patch-clamp studies were conducted on inside-out patches of the apical membrane of IMCD cells in primary culture. Previous studies showed that the Na^+ current is inhibited by amiloride, atrial natriuretic peptide, and cGMP (Light et al. 1989. *Science.* 243:383). GTPγS (10^{-4} M) in the bath increased the single-channel open probability (P_o) from 0.17 to 0.47 ($n = 9$; $P < 0.01$). In contrast, GDPβS (10^{-4} M) reduced P_o from 0.57 to 0.25 ($n = 7$; $P < 0.05$). Pertussis toxin (PTX; 100 ng/ml) reduced P_o from 0.57 to 0 ($n = 9$; $P < 0.01$). GTPγS but not GTP reversed PTX inhibition. In PTX-treated patches exogenous α_{i-3}^* (2 pM) increased P_o to 46% of the pre-PTX level and 200 pM raised P_o to 111% of the pre-PTX value. Heat-inactivated α_{i-3}^* had no affect. α_o^* also activated the channel but at a threshold concentration of 200 pM. In contrast, G_s^* (200 pM) did not change P_o. ATPγS, phorbol esters, and exogenous cGMP-dependent protein kinase inhibited channel activity: thus α_{i-3}^* does not activate the channel by stimulating a kinase. Our data show that α_{i-3} regulates Na^+ influx across the apical membrane. Because channels are active in the absence of exogenous agonist, there may be a constitutively active G protein in the apical membrane, or membrane-derived autacoids may bind to apical receptors and stimulate the G protein. Alternatively, an effector activated by a G protein complex in the basolateral membrane may serve as an intracellular agonist and activate the G protein in the apical membrane. [Supported by NIH grant DK-34533 and The Hitchcock Foundation.]

68. Effects of Insulin, Phosphatase, and Cholera Toxin on a Ca^{2+}-dependent Chloride Channel in a Distal Nephron Cell Line (A6) YOSHINORI MARUNAKA* and DOUGLAS C. EATON, *Department of Physiology, Emory University School of Medicine, Atlanta, Georgia*

To examine regulation of a renal chloride channel, we studied the effects of varying $[Ca^{++}]_i$ and the effects of insulin, phosphatase, or cholera toxin on single 3 pS Cl channels (originally observed in cell-attached patches from the apical membrane of confluent A6 cells cultured on permeable supports for 10–14 d). The open probability was very low (<0.1) and voltage dependent (increasing with depolarization). The I-V curve for channels from cell-attached patches strongly rectified with no detectable inward current at potentials more negative than E_{Cl} but with increasing outward cur-

rent above E_{Cl}. The outward rectification without any detectable inward current is due to the small single-channel (unit) conductance for inward currents. The unit conductance for inward currents in inside-out patches changed in a $[Ca^{++}]_i$-dependent manner; i.e., the unit conductances at 1, 10, 100, and 800 μM $[Ca^{++}]_i$ were respectively <0.6, 0.9, 1.7, and 3.5 pS. On the other hand, the unit conductance for outward currents was independent of $[Ca^{++}]_i$ (3.2 ~ 3.5 pS). After adding insulin to the cell, the sensitivity of the channel to $[Ca^{++}]_i$ increased 100-fold compared with cells without insulin treatment; i.e., insulin increased the unit conductance for inward currents in cell-attached patches and induced inward currents (Cl^- secretion) at the normal resting potential. Application of phosphatase to the intracellular surface of the channel mimics the action of insulin; i.e., phosphatase increased the unit conductance for inward currents to 1.2 pS from ≤0.6 pS at 1 μM $[Ca^{++}]_i$. Application of cholera toxin activated the channel. [Supported by NKF grant to Y.M. and NIH grants DK-37963 and DK-38830 to D.C.E.]

69. Insulin-like Growth Factor 1–stimulated Na^+ Transport in A6 Cells: A G Protein-mediated Response BONNIE L. BLAZER-YOST and MALCOLM COX, *Department of Medicine, University of Pennsylvania School of Medicine and Veterans Administration Medical Center, Philadelphia, Pennsylvania*

The A6 cell line derived from the kidney of the amphibian *Xenopus laevis* is a well characterized in vitro model of the mammalian distal nephron. Aldosterone, vasopressin, and insulin are known to stimulate net mucosal to serosal Na^+ flux across the high-resistance epithelium formed by confluent A6 cell monolayers. We have found that physiologically relevant (nanomolar) concentrations of insulin-like growth factor 1 (IGF1) also stimulate net mucosal to serosal Na^+ flux (measured as short circuit current) in A6 cell monolayers. This response occurs within minutes and is blocked by 10^{-5} M amiloride, a specific inhibitor of the conductive Na^+ channel. We have previously demonstrated the presence of specific IGF1 receptors in another amphibian epithelium: the urinary bladder of the toad *Bufo marinus* (Blazer-Yost et al. 1989. *FASEB J.* 3:4394). The stimulation of A6 cell Na^+ transport by nanomolar concentrations of IGF1 is consistent with the presence of specific IGF1 receptors in this epithelium as well. Preincubation of A6 cells with pertussis toxin (200 ng/ml) for either 3 or 16 h inhibits IGF1-stimulated Na^+ transport (51 ± 7% $n = 6$). Pertussis toxin is well known to ADP-ribosylate a class of guanine nucleotide–binding proteins (G proteins). Therefore, IGF1-stimulated Na^+ transport in A6 cells appears to involve a pertussis toxin–sensitive G protein. Whether this G protein has a traditional role in signal transduction or modulates the epithelial conductive Na^+ channel remains to be determined. [Supported by a V.A. Merit Review grant and by a University of Pennsylvania Biomedical Research Support grant.]

70. Dual Modulation of Cl^- Conductance by Nucleotides in Pancreatic and Parotid Zymogen Granules FRANK THÉVENOD,* KENNETH GASSER,* ADAM GOLDSMITH,* and ULRICH HOPFER, *Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio*

A regulated Cl^- conductance pathway in zymogen granules (ZG) may contribute to overall activation of Cl^- permeability during hormone-stimulated secretion of primary fluid and enzymes in exocrine glands. We have investigated the role of nucleotides and analogues in modulating Cl^- conductance in pancreatic and parotid ZG. Cl^- conductance was assayed by measuring the rate of osmotic lysis of ZG suspended in 150 mM KCl buffer after addition of the electrogenic K^+ ionophore valinomycin. GTP, GTP-γ-S, ATP-γ-S, UTP, CTP, and ITP (1–500 μM) reduced Cl^- conductance up to twofold. In contrast, AMP-PCP and AMP-PNP stimulated Cl^- conductance up to threefold. ATP reduced Cl^- conductance at 1 μM and stimulated above 50 μM. GDP-β-S was inhibitory at ~5 mM Mg and stimulated at ~0.1 mM Mg. AlF^- was stimulatory up to twofold at 15 mM F^-; higher F^- concentrations reversed stimulation. When ZG were preincubated with activated pertussis toxin (PTX), washed through a G25-300 Sephadex column and assayed for Cl^- conductance, it was decreased 1.5-fold, and even 2.3-fold with GTP (100 μM) in the medium, as compared with controls without PTX. Surprisingly, PTX reduced Cl^- conductance independent of NAD^+. The data suggest

a dual pattern of modulation of the Cl^- conductance activity in pancreatic and parotid ZG that cannot be explained by classical G proteins only. [Supported by a grant from the Max-Kade-Foundation and by the CF Foundation.]

71. The Apparent Rate of Spontaneous G Protein Activation in *Limulus* Photoreceptors Is Extremely Low JOHN LISMAN, MARC GOLDRING,* PHYLLIS ROBINSON,* and ALFREDO KIRKWOOD,* *Department of Biology, Brandeis University, Waltham, Massachusetts*

An active receptor molecule catalyzes the exchange of GTP for GDP on G protein, but such exchange also occurs spontaneously at a low rate. For purified transducin the rate is 3×10^{-5}/s (Wessling-Resnick and Johnson. 1987. *J. Biol. Chem.* 262:3697). In *Limulus* photoreceptors, an easily detectable electrical event called a quantum bump is generated by the activation of G protein. Spontaneous events also come from other sources; the total rate is ~0.3/s. Analysis of the size distribution of spontaneous events (at 18°C) suggests that 10–30% of spontaneous bumps have the size expected of bumps originating at the G protein, as opposed to at rhodopsin. From this rate, together with the fact that these photoreceptors contain $\sim 10^9$ rhodopsin molecules and 0.1 G protein/rhodopsin, it can be calculated that the apparent rate constant for spontaneous G protein activation is on the order of 10^{-9}/s. This is more than 4 orders of magnitude lower than that measured for transducin. It is unclear what accounts for this large discrepancy. One mechanism that could substantially lower the probability that spontaneous activation of G protein would trigger an electrical event, would be for the enzyme activated by transducin to function as a "coincidence detector," requiring the simultaneous presence of two activated G proteins in order to become active. The reduction in the rate of spontaneous events achieved by such a mechanism would lower the "noise" and thereby enhance the ability of these cells to detect dim light.

72. G Proteins Mediate Immunoglobulin E Receptor-activated Signal Transduction Pathways in Rat Basophilic Leukemia Cells V. NARASIMHAN,* G. LABRECQUE,* D. HOLOWKA,* and B. BAIRD,* *Cornell University, Ithaca, New York* (Sponsor: C. Fewtrell)

Aggregation of high affinity receptors for immunoglobulin E (IgE) by antigen-mediated cross-linking leads to degranulation of mast cells in the allergic response. We have previously shown that IgE receptor activation of a Ca^{2+} influx pathway in the rat basophilic leukemia (RBL) mast cell line is mediated by a cholera toxin–sensitive G protein and does not depend on activation of phospholipase C (Narashimhan et al. 1988. *J. Biol. Chem.* 263:19626). Receptor-mediated activation of both phospholipase C and A2 (PLC and PLA2) is mediated by G proteins, as evidenced by the inhibition of these pathways by GDPβS in streptolysin O–permeabilized cells. Low concentrations of GTPγS (0.1 μM) synergize with antigen to activate PLA2 in these permeabilized cells at micromolar free Ca^{2+}, and this activity can be reconstituted with exogenous secreted forms of PLA2 after chemical inactivation of the endogenous PLA2. Neither of these receptor-mediated activities in permeabilized cells is sensitive to cholera toxin or pertussis toxin pretreatment of the intact cells, and antigen-stimulated PLA2 is insensitive to pretreatment with phorbol myristate acetate under conditions where activation of PLC is inhibited. IgE receptor activation of K^+ efflux is inhibited ~50% by pertussis toxin pretreatment, suggesting that at least one pathway for stimulated K^+ efflux is mediated by a pertussis toxin–sensitive G protein. These results are consistent with a model in which aggregated IgE receptors activate at least four different G proteins, each of which mediates the activation of a different effector pathway. [Research supported by the NIH and the Cornell Biotechnology Program which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, and the US Army Research Office.]

73. Ion Channel Subconductance States: Analysis and Structure-Function Implications A. M. J. VANDONGEN* and A. M. BROWN, *Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas*

Functional studies of ion channels using different techniques have led to the concept of the unitary conductance: channels can exist in two states, open or closed. In the open state the permeability is constant and characteristic for a given channel. Single-channel measurements initially confirmed this view, although presently the existence of subconductance levels is documented for several channels. As standard idealization algorithms are not suitable for the analysis of subconductance levels, a new algorithm was developed. It uses the first derivative to detect transitions between levels, after which the amplitudes and durations of all levels are estimated. No a priori information is required: necessary parameters are obtained from a part of the recording that contains no openings. Tests using simulated data showed that the algorithm reliably idealizes complicated signals; in the absence of noise the idealization is perfect. Using this algorithm, subconductance levels were shown to exist in (*a*) a Na^+ channel, (*b*) several G_o-gated K^+ channels from rat hippocampus, (*c*) a glutamate receptor-channel, and (*d*) a delayed rectifier K^+ channel which we have recently cloned (*a*, *c*, and *d* are rat brain channels expressed in *Xenopus* oocytes). Maximum-likelihood analysis of the amplitudes indicated that the Na^+ channel has four equidistant subconductance levels. The current structural model for the sodium channel has four homologous domains, each of which consists of six transmembrane α-helices. To account for the existence of four equidistant subconductance levels, the following is proposed: (*a*) all four domains contribute equally to permeation, (*b*) this contribution is dynamic, i.e., a negative charge that can move into and out of the pore, and (*c*) S2 and S3 are the best candidates for channel lining, since they both have a negative charge which is conserved amongst all voltage-gated channel cloned so far.

74. "Perforated Patch Recording" from Pancreatic Islet B Cells LEE C. FALKE, KEVIN D. GILLIS, DAVID M. PRESSEL, and STANLEY MISLER, *The Jewish Hospital, Washington University Medical Center, St. Louis, Missouri*

Recently Horn and Marty (1988. *J. Gen. Physiol.* 92:145) described a "perforated patch" recording technique in which the pore-forming antibiotic nystatin is used to obtain low-resistance access to the cytoplasm while minimizing exchange of the pipette solution and the cytoplasm. We have applied this technique to rat and human pancreatic islet B cells; it permits current-clamp recording of metabolite-induced electrical activity, membrane resistance measurements, and voltage-clamp recording of usually labile Ca^{2+} currents for hours, from both single cells and small clumps of cells. B cells with resting potentials of -55 to -60 mV in 0–3 mM glucose repeatedly depolarize to plateau potentials of -45 to -40 mV and display action potentials (AP) peaking at -20 to 0 mV on raising bath glucose to 8–10 mM, or after adding the K^+(ATP) channel blocker tolbutamide (20 μM). Cells hyperpolarize to ≥ -70 mV on further addition of the oxidative inhibitor NaN_3 (3 mM) or the K^+(ATP) channel enhancer diazoxide (200 μM). After addition of 15 mM Ba^{2+} and 20 mM TEA^+ to the Ringer's bath, rat B cells display sustained inward currents on depolarization from a holding potential (V_h) of -90 mV to clamping potentials (V_c's) positive to -20 mV; these currents are also enhanced by BAY K 8644, suggesting that they are composed of "L-type" Ca^{2+} channels. In human B cells, a transient BAY K-insensitive component of inward current is also seen at V_c's positive to -40 mV, after activation from a V_h of -90 mV but not -60 mV, which suggests underlying "T-type" Ca^{2+} channels. These results suggest that "perforated patch recording" may facilitate studies of the electrical responses to hormones and transmitters operating through second messenger cascades. [Supported by a NIH grant DK-37380 and the American Diabetes Association, Inc.]

75. Inhibition of Epithelial Anion Channels by HEPES and Related Buffers J. A. TABCHARANI* and J. W. HANRAHAN,* *Department of Physiology, McGill University, Montreal, Quebec* (Sponsor: L. Reuss)

The effects of pH buffers on single outwardly rectifying anion channels have been studied using patches excised from PANC-1, an epithelial cell line derived from human pancreatic duct. *I/V* curves were determined in the nominal absence of buffer and in the presence of substituted taurines (HEPES, MES, and BES), glycines (glycylglycine, bicine, and tricine) and Tris. $HEPES_i$ (10

mM) reduced open channel conductance at the reversal potential by ~30%. Inhibition had voltage-dependent and -independent components which probably correspond to fast block by the anionic and zwitterionic (protonated) forms of the buffer, respectively. Voltage dependence at positive membrane potentials could be fitted with a Woodhull-type model in which the $K_d(0)$ = 6.1 mM $HEPES^-$ and the electrical distance from the inner surface of the membrane is ~0.45. The voltage-independent component was studied by varying [total HEPES] at constant [$HEPES^-$] and vice versa. Voltage-independent block displayed saturation and a K_d of ~20 mM. HEPES effects were studied with pH-matched controls because Cl conductance declined slightly with increasing pH independently of the buffer. Neither hydroxyethylpiperazine nor ethanesulfonate reduced conductance, suggesting both parts of the HEPES molecule are needed for channel inhibition. MES reduced conductance by the same amount as HEPES while BES and taurine had no effect. This indicates the ability of a buffer to block does not depend on having a particular aliphatic ring, but suggests some heterocyclic group is required. The length of the alkylsulfonate chain is not critical because conductances measured with MOPS and MES were identical. Substituted glycine buffers and Tris did not affect conductance although the latter induced additional brief closures. Conclusions: several common aminosulfonates reduce the conductance of this epithelial anion channel. BES (pK_a = 7.15), tricine (pK_a = 8.15), and bicine (pK_a = 8.35) are the most suitable buffers when studying its conductive properties. [Supported by the U.S. and Canadian CF Foundations and by the M.R.C. (Canada).]

76. Whole-Cell Currents in Cultured Outer Medullary Collecting Duct Cells C. PAPPAS,* A. OYLER,* and B. KOEPPEN, *Departments of Medicine and Physiology, University of Connecticut Health Center, Farmington, Connecticut*

Studies were done to characterize the whole-cell currents and conductances of cultured outer medullary collecting duct (OMCD) cells; a model epithelium for electrogenic H^+ secretion. In accordance with earlier microelectrode measurements, a Cl^--dependent conductance was found in all cells. In addition, an outward current, activated by positive (depolarizing) pulses, was observed in a small number (20%) of the cells. In general, cells expressing this depolarization-activated outward current had a higher input resistance (986 ± 84 MΩ vs. 452 ± 50 MΩ), and a higher resting membrane voltage (−61.2 ± 8.0 mV vs. −33.3 ± 4.0 mV), when compared with the majority of the cells not expressing this current. In some cells it was possible to distinguish single-channel events at positive (depolarizing) membrane voltages. The channel had a unit conductance of 140 ± 2 pS, and the open probability was 2.3 ± 0.6% at 0 mV and 12.8 ± 0.1% at +40 mV. In preliminary studies we have attempted to identify the current-carrying ion. The current was present when all Cl^- was removed from the bathing medium, and tetraethylammonium (TEA) added to the bathing medium at 1 mM reduced the open probability at +40 mV from 12.8 to 2.0%. Based on the single-channel conductance, activation by depolarizing voltages, and sensitivity to TEA, we postulate that the outward current is carried by K^+ via a "maxi-K^+ channel." Because the channel is only open at positive membrane voltages, it is not expected to contribute significantly to the electrical properties of the cell at the resting membrane voltage. [Supported by NIH grant DK-32489; B.K. is an established investigator of the American Heart Association.]

77. Single-Channel Properties of a Chloride Channel from Lobster Axon Vesicles GERGELY L. LUKACS* and EDWARD MOCZYDLOWSKI, *Departments of Pharmacology and Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut*

Membrane vesicles from lobster walking leg axons were prepared according to Correa et al. (1987. *Biochim. Biophys. Acta.* 897:406). Single chloride-selective channels from this preparation were incorporated into bilayers (80% PE/20% PC in decane) in the presence of symmetrical 10 mM Tris-Cl, pH 7.4 and 0.1 M NaCl at 22°C. When vesicles were added to the *cis* side (*trans* = ground), Cl channels incorporated in a preferred orientation as determined by slightly rectifying *I-V* behavior

of the open channel and weak voltage dependence of channel gating, with P_o increasing from 0.3 at −70 mV to 0.6 at +70 mV. In symmetrical 0.1 M NaCl, the unitary conductance of the fully open state was 18.7 ± 1.6 pS at positive voltages and 14.2 ± 1.9 pS in the negative voltage range (±SD, n = 10). The unitary conductance appeared to saturate with increasing symmetrical [NaCl] with an apparent $K_{0.5}$ value of 96 mM and a maximal conductance of 32 pS. Using a NaCl gradient of 0.2 M *cis*/0.1 M *trans*, the permeability ratio of Cl^- to that of Na^+ was 14.2 ± 0.6 (n = 7). Under biionic conditions (*trans* 100 mM NaCl/*cis* 100 mM Na-anion), the relative permeability for halide ions increases with ionic radius and the channel carries gluconate$^-$ current, suggesting a large pore diameter. The selectivity sequence is $I^- > Br^- > NO_3^- > Cl^- > HCO_3^- > F^-$ > gluconate$^-$ > $H_2PO_4^- > SO_4^{2-}$. The chloride transport inhibitor, NPPB (5-nitro-2-[3-phenylpropylamino]benzoic acid), induces a reversible, voltage-dependent decrease in the unitary current with a K_d of 149 ± 19 μM (n = 3) at 0 mV and an effective valence of 0.28 ± 0.08. The disulfonic stilbene derivative, SITS, inhibits the channel preferentially from the *trans* side. This information may be useful in assessing the contribution of chloride conductance to the electrophysiological properties of crustacean nerve. [NIH grants HL-38156, AR-38796, and an Established Investigator award from the American Heart Association.]

78. Patch-Clamp Studies of Single Fusion Events Caused by the Influenza Virus Fusion Protein A. E. SPRUCE, A. IWATA, J. M. WHITE, and W. ALMERS, *Department of Physiology and Biophysics, University of Washington, Seattle, Washington; and the Department of Pharmacology, University of California, San Francisco, California*

3T3-HA-b fibroblasts (FBs) expressing the influenza fusion protein (HA) provide a convenient model to study membrane fusion (Doxsey et al. 1985. *J. Cell Biol.* 101:19). FBs were made fusion competent by mild trypsinization, and decorated with human erythrocytes (RBCs). Activation of HA by pH 4.8 triggers fusion as demonstrated by transfer of fluorescence into FBs from RBC ghosts loaded with Lucifer Yellow. In whole-cell recordings, fusion-competent FBs decorated with one fresh RBC had conductances (G) of 0.09 ± 0.02 nS and capacitances (C) of 14 ± 1 pF (±SEM, n = 11). Within 63 ± 5 s after pH 4.8, C (the imaginary part of the admittance of the FB to a sinusoidal voltage, *Im*) increased abruptly, then more slowly (over 91 ± 15 s), ultimately by 0.92 ± 0.03 pF. During this time, the real component of the admittance (*Re*) increased only transiently as expected if the fusion pore connecting RBC and FB dilates slowly (Breckenridge and Almers. 1987. *Nature.* 328:814). RBCs decorating FBs had G = 0.14 ± 0.03 nS and C = 0.90 ± 0.02 pF (n = 12). The C increase in FBs reflects fusion with the RBC since (*a*) it is not seen in the absence of RBCs and (*b*) it equals the RBC capacitance. Fusion was not seen at pH 7.4 nor in nontrypsinized FBs, indicating the requirement for activated HA. From the initial deflections in *Re* and *Im* we calculate the fusion pore conductance to be 554 ± 78 pS. Therefore, HA-mediated fusion, like exocytosis, is nonleaky (G increased insignificantly by 40 ± 30 pS) and starts with a small pore but exocytosis is 1,000 times faster (Breckenridge and Almers, 1987). [Supported by NIH grant GM-39520].

79. A Potassium-selective Ion Channel in Gastric Smooth Muscle Cells Is Activated by Flow and Ca^{2+} at the Extracellular Surface MICHAEL T. KIRBER,* RICHARD W. ORDWAY,* JOHN V. WALSH, JR., and JOSHUA J. SINGER, *Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts*

A K^+ channel, which appears to be sensitive to both extracellular Ca^{2+} and flow of solution at the extracellular surface of the membrane, has been studied in freshly dissociated smooth muscle cells from the stomach of the toad *Bufo marinus*. In the absence of external Ca^{2+} (2 mM EGTA), channel openings were very infrequent, whereas in the presence of external Ca^{2+} (2 mM EGTA), channel openings were very infrequent, whereas in the presence of external Ca^2 (0 mM EGTA) substantial channel activity was observed. The channel was K^+ selective and had a slope conductance of 20 pS (nominally 3 mM $[K^+]o$/140 mM $[K^+]_i$). To test the effects of flow on the channel, a solution identical to that already bathing the extracellular surface of an outside-out patch (5 mM EGTA in the

patch pipette) was applied from a pressure ejection pipette with a tip diameter of ~1 μm. In this way application of pressure to the ejection pipette did not change the solution bathing the patch but did induce flow. Flow generated in this way greatly increased the probability that the channel was open (P_o) when Ca^{2+} was present in the bathing and application solutions. In contrast, flow generated in the absence of Ca^{2+} (2 mM EGTA) in bathing and application pipette solutions did not increase P_o; also, the application of Ca^{2+}-free (2 mM EGTA) solutions by pressure ejection decreased P_o when the bath contained Ca^{2+}. Furthermore, the increases in P_o generated by the application of Ca^{2+}-containing bath solution could be overridden by the application of Ca^{2+}-free (2 mM EGTA) solutions through a second pressure ejection pipette with greater flow. Previously we have described stretch-activated channels in the same cells (Kirber et al. 1988. *Pflügers Archiv.* 412:339–345). In contrast to the channels described here, the stretch-activated channels were cation selective, did not require Ca^{2+} at the extracellular surface of the channel for activation, and were activated by suction applied to the patch pipette, and not by flow at the extracellular surface. [Supported by NSF grant DCB-8511674 and NIH grant DK-31620.]

80. Fatty Acids Directly Activate Large Conductance, Calcium-activated K^+ Channels in Pulmonary Artery Smooth Muscle Cells from Rabbit RICHARD W. ORDWAY,* LUCIE H. CLAPP,* ALISON M. GURNEY, JOSHUA J. SINGER, and JOHN V. WALSH, JR., *Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts; and Department of Pharmacology, United Medical and Dental Schools, St. Thomas' Campus, London, England*

The effects of fatty acids (FAs) on smooth muscle cells isolated from rabbit pulmonary artery were studied using patch-clamp techniques. Both arachidonic acid (AA) and other FAs that, unlike AA, are not substrates for cyclooxygenase and lipoxygenase enzymes, activated the large conductance calcium-activated K^+ channel (CAK channel) in excised, inside-out membrane patches. This CAK channel exhibited several characteristics of large conductance CAK channels, including: increased activity with increasingly positive membrane potentials, activation by Ca^{++} at the cytosolic face of the membrane, and a unitary conductance of ~200 pS (taken from outward currents; 20 mM $[K^+]_o$/130 mM $[K^+]_i$). FAs were applied to patches by pressure ejection from a micropipette. CAK channel activation by FAs was observed in the absence of Ca^{++} (5 mM EGTA on both sides of the membrane) at membrane potentials more positive than 0 mV. The activity of CAK channels was increased by AA (20:4 *cis*-5,8,11,14) (20 μM), linoelaidic acid (18:2 *trans*-9,12) (20–40 μM), and myristic acid (14:0) (20 μM), the latter two of which are not substrates for the oxygenases. Since FA activation of this channel occurred in the absence of Ca^{++} and nucleotides, and was elicited by FAs that are not converted to active metabolites by known oxygenases, we conclude that FAs activate these channels directly rather than through cyclooxygenase, lipoxygenase, or NADPH-dependent cytochrome P450 pathways. These results provide a second example of direct activation of smooth muscle K^+ channels by FAs. Previously, in gastric smooth muscle cells isolated from the toad, *Bufo marinus*, we found that FAs directly activate a different K^+ channel (23 pS; 20 mM $[K^+]_o$/130 mM $[K^+]_i$) in excised patches, both outside-out and inside-out, and in cell-attached patches. (Ordway et al. *Science.* In press). [Supported by NSF grant DCB-8819750.]

81. Stretch-activated Ion Channels in Mammalian Vascular Smooth Muscle Cells MICHAEL T. KIRBER,* LUCIE H. CLAPP,* ALISON M. GURNEY, JOHN V. WALSH, JR., and JOSHUA J. SINGER, *Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts; and Department of Pharmacology, United Medical and Dental Schools, St. Thomas' Campus, London, England*

Patch-clamp techniques were used to identify mechanically activated ion channels in smooth muscle cells freshly dissociated from rabbit pulmonary artery. In cell-attached patches these channels are inactive until suction is applied to the patch pipette, which elicits channel openings that become

more frequent as suction is increased. When the cells are bathed in a physiological saline solutoin, and pipette solutions contain high Na^+ and low K^+ concentrations (137 mM Na^+, 3 mM K^+, 0 mM Ca^{2+}, 2 mM EGTA), stretch-activated channels conduct inward currents in cell-attached patches when the pipette potential is zero. The current-voltage relationship shows inward rectification and the slope conductance measured from the inward currents is ~40 pS. This conductance is smaller than that of stretch-activated channels in gastric smooth muscle cells isolated from toad (Kirber et al. 1988. *Pflügers Archiv.* 412:339–345). In addition, the apparent density of these channels in the cell membrane is lower than that which we found in the toad cells. Changes in membrane potential did not elicit channel openings, but large positive potentials favor long duration channel openings when suction is applied. Our results indicate that when these vascular cells are at normal resting potential, inward, depolarizing currents are conducted through the stretch-activated channels. These channels may play a role in myogenic regulation of vascular tone. [Supported by NSF (DCB-8511674) and NIH (DK-31620).]

82. Ionic Currents in Smooth Myocytes of the Pregnant Rat Uterus M. YOSHINO, S. Y. WANG, and C. Y. KAO, *Department of Pharmacology, State University of New York Downstate Medical Center, Brooklyn, New York*

Smooth myocytes from 17–21 d pregnant but nonparturient rat uteri have been dissociated with collagenase, and studied within hours with the tight-seal patch-clamp method in the whole-cell mode. In 3 mM $[Ca^{2+}]_o$, inward and outward currents show a great deal overlap at ~22°C. The inward current is usually small, and is enlarged when the outward current is reduced by bath-applied TEA (30 mM) or blocked by pipette-applied Cs^{2+}. From a holding potential of −60 mV, the inward current is first observed at ~40 mV, reaches a maximum at ~0 mV, and reverses at ~ +80 mV. Its amplitude varies directly with $[Ca^{2+}]_o$; it is blocked by nisoldipine (2 μM), and Co^{2+} (5 mM), and increased and prolonged by BAY K 8644 (2 μM) and Ba^{2+} (3 mM). It attains a maximum at ~10 ms. Inactivation has a τ of ~40 ms, and is partly mediated by $[Ca^{2+}]_i$. The outward current rises gradually and smoothly, with little initial hump. It has a reveral potential of ~65 mV. In 0 or 30 mM $[Ca^{2+}]$, when the inward current is abolished or increased, the outward current remains the same. These observations show that in the pregnant and nonparturient myometrium, Ca^{2+} carries a significant part of the inward current, and that there is little Ca^{2+}-activated potassium conductance. [Supported by NICHD grant 00378.]

83. Actions of C-6–modified Tetrodotoxin on Frog Skeletal Muscle Fibers L. YANG, S. L. HU, C. Y. KAO, and T. YASUMOTO, *Department of Pharmacology, State University of New York Downstate Medical Center, Brooklyn, New York; and Department of Food Chemistry, Faculty of Agriculture, Tohoku University, Sendai 980, Japan*

In tetrodotoxin (TTX), C-6 is positioned almost directly opposite the guanidinium function. Several natural analogues with modifications on C-6 have been isolated and characterized. We have compared the actions of 6-epi TTX and 11-deoxy TTX with TTX. In 6-epi TTX, the −H and −OH are reversed from those in TTX. In 11-deoxy TTX, the hydroxymethyl is replaced by a methyl function. Short segments of single fibers from the semitendonosus have been voltage clamped in the vaseline gap method. The external solution contained only 40 mM Na^+ to reduce I_{NA} and errors caused by the series resistance. For estimating potencies, Cs^{2+} is allowed to diffuse into the fiber from the cut-ends to block I_K. At pH 7.25, the ED_{50} are: 3.5–4 nM for TTX, 132 nM for 6-epi TTX, and 400 nM for 11-deoxy TTX. These findings suggest that both the −OH groups on C-6 and in the C-11 hydroxymethyl function play a role in the binding of TTX to its receptor site, most probably through some hydrogen bonding. In fibers in which I_K is intact, 6-epi TTX and 11-deoxy TTX significantly slow the "activation" phase of the outward current. There is an appreciable reduction in the I_K at 10 ms, which is not present at 20 ms. This complex effect on the outward current is probably due to the presence of a slow residual and overlapping inward current originating possibly from T-tubular sodium channels. [Supported by NINCDS grant 14551, and Army contract DAMD17-87-C-7094.]

84. Trypsin Activation and Quinidine Block of Atrial Muscarinic K^+ Channels GLENN E. KIRSCH* and ARTHUR M. BROWN, *Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas*

The atrial muscarinic K^+ channel normally is opened by the activated G protein, G_k. Assuming that an inactivating particle keeps the channel closed, several protein-modifying agents including trypsin, papain, glyoxal, and phenylglyoxal, which remove Na^+ channel inactivation, were tested. K^+ channels were studied in inside-out excised membrane patches from primary cultures of neonatal rat atrial cells using the gigaseal patch-clamp method. Of the agents tested, only trypsin-activated muscarinic K^+ channels and it did so irreversibly. Trypsin was effective in the absence of muscarinic agonist or intracellular Mg^{2+} and GTP. Since trypsin is known to inactivate G proteins, the effect was probably on the K^+ channel or a structure closely associated with it. Heat-denatured trypsin was ineffective and trypsin inhibitor blocked the effect. Trypsin activation produced single-channel currents whose inward rectification, single-channel conductance, mean open time, and burst duration were indistinguishable from muscarinic activation. The antiarrhythmic agent, quinidine, which has anticholinergic side effects, was found to block trypsin-activated channels at therapeutic concentrations. This result shows that the anticholinergic effects of quinidine can be explained by a direct interaction with the muscarinic K^+ channel rather than through interactions with the muscarinic receptor or G_k. We conclude that the muscarinic atrial K^+ channel contains a trypsin-sensitive, inhibitory gating mechanism, which normally holds the channel closed in the absence of activated G_k, as well as a trypsin-insensitive binding site for blockers such as quinidine. The inhibitory gate is physically distinct from the gate that mediates bursting and must contain at least one trypsin cleavage point accessible from the cytoplasmic surface of the cell membrane. [Supported by the American Heart Association (Texas Affiliate) and NIH grants HL-25143 and HL-33662.]

85. Reconstitution of a Calcium-activated Potassium Channel in Basolateral Membranes of Rabbit Colonocytes into Planar Lipid Bilayers KLAUS TURNHEIM,* JAMES COSTANTIN,* STEPHANIE CHAN,* and STANLEY G. SCHULTZ, *Department of Physiology and Cell Biology, University of Texas Medical School at Houston, Houston, Texas*

A highly enriched preparation of basolateral membrane vesicles was isolated from rabbit distal colon surface epithelial cells using the method described by Wiener, Turnheim, and Van Os (*J. Membrane Biol.* In press) and incorporated into planar lipid bilayers. With very few exceptions (≪1%) the only channel activity observed was that of a high conductance, Ca^{2+}-activated K^+ channel. This channel is highly selective for K^+ over Na^+ and Cl^-, displays voltage-gating similar to "maxi" K(Ca) channels found in other cell membranes, and kinetic analyses are consistent with the notion that K^+ diffusion through the channel involves "single filing" ("multi-occupancy"). Channel activity is inhibited by the venom from the scorpion *Leiurus quinquestriatus*, Ba^{2+}, quinine, and trifluoperizine. If the Ca^{2+} sensitivity and voltage-dependence of K(Ca) channels in the basolateral membranes of intact cells resemble those of the reconstituted channel, our data suggest that these channels would be inactive under physiological conditions. Thus, our findings raise two perplexing questions. First, what role might these channels play in the functions of these cells? And, second, why could we not detect other K^+ channels that are responsible for the high K^+ conductance of the basolateral membranes of these cells under physiological conditions? [Supported by NIH grant DK-37620.]

86. Ion Channel Activity in B Lymphocytes Mediated by Interleukin-4 FRANCES V. McCANN, DAVID C. McCARTHY,* and RANDOLPH J. NOELLE,* *Departments of Physiology and Microbiology, Dartmouth Medical School, Hanover, New Hampshire*

Interleukin-4 (IL4), a lymphokine, exercises a potent influence on the growth and differentiation of B lymphocytes. IL4 has been shown to induce an increase in B cell size, augment the transition of B cells stimulated by anti-immunoglobulin to enter S phase, increase class II MHC and FcRε expression, modify the Ig isotype, and enhance the expression of IgG_1 and IgE in lipopolysaccharide-stimulated B cells. Using the patch electrode technique, we have demonstrated differences in the expression of specific ion channel currents in IL4-activated murine B lymphocytes as compared

with those recorded in resting murine B lymphocytes. Ion channels were recorded in 83% of i/o (inside/out) patches in IL4-treated cells ($n = 93$) but were found in <60% of i/o patches on untreated cells ($n = 267$). An inward rectifying K^+ channel with a single-channel conductance of 30.4 ± 1.17 pS ($n = 11$) was recorded in over 21% of the patches on IL4-treated cells ($n = 93$) but was found in <1% of the patches on untreated cells ($n = 267$). This channel exhibited voltage-gated kinetics with multiple open and closed states and was active in both the cell-attached and i/o patch configurations. Although selectively permeable to K^+ in physiological gradients ($P_K/P_{Na} = 17$), the channel conducted Na^+ when 140 mM Na^+ was substituted for K^+ on the intracellular side of the membrane. IL4 also enhanced the activity of a large conductance anion channel, the most predominant type of ion channel expressed in freshly isolated murine B lymphocytes (McCann et al., unpublished observations). In untreated resting B lymphocytes this anion channel remains inactive in cell-attached patches, becoming active only upon patch excision (McCann et al. 1989. *Cellular Signalling.* 1:31). Conversely, we have shown that anion channels in IL4-treated cells are active in both of the patch configurations. These electrophysiological changes mediated by IL4 suggest a functional role for both the inward rectifying K^+ channel and the large conductance anion channel in B cell activation. [Supported by NIH grant GM-37767 and the Norris Cotton Cancer Center Support grant CA23018.]

87. Parathyroid Hormone–stimulated Calcium Channels in Kidney [CAL + DCT] Cells

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Parathyroid hormone (PTH) increases renal calcium absorption in cortical thick ascending limbs (CAL) and distal convoluted tubules (DCT). PTH-sensitive cells from these nephron sites were isolated from mouse kidneys and grown in culture. Intracellular calcium ($[Ca^{2+}]_i$) was measured at 23–25°C with fura-2. bPTH[1-34] (10 nM) led to a slow (15-min onset, 30-min maximum) increase in $[Ca^{2+}]_i$ from resting levels of 80 nM to sustained values of 250 nM. Addition of 1 mM dibutyryl cAMP mimicked the PTH response; removal of extracellular calcium inhibited the PTH effect. Addition of Bay K 8644 (50 μM) after the PTH-induced increase in $[Ca^{2+}]_i$ reached plateau levels resulted in an immediate additional rise in $[Ca^{2+}]_i$. BAY K 8644 treatment alone had little or no effect on $[Ca^{2+}]_i$. Addition of PTH after BAY K 8644 caused a monotonic increase in $[Ca^{2+}]_i$ to maximal levels. Nifedipine (50 μM) blocked the PTH stimulation of $[Ca^{2+}]_i$ but had no effect alone. These data suggest that PTH may lead to the insertion or activation of calcium channels. Colchicine (1 μM), abolished the effects of PTH and of PTH + BAY K 8644 on $[Ca^{2+}]_i$. Cellular subapical vesicles were loaded with acridine orange. PTH caused a time-dependent decline in the number of labeled vesicles. The decrease in acridine fluorescence followed the same time-course as the PTH-induced increase in $[Ca^{2+}]_i$. Acridine fluorescence was virtually unchanged in the absence of PTH. We conclude: (*a*) PTH leads to the insertion of dihydropyridine-sensitive calcium channels in [CAL + DCT] kidney cells, (*b*) cAMP mediates, at least in part, the increase in $[Ca^{2+}]_i$, (*c*) the increase in $[Ca^{2+}]_i$ represents an influx of extracellular calcium, (*d*) once inserted or partially activated, apical calcium channels can be fully activated by BAY K 8644, and (*e*) these calcium channels appear to be stored in subapical vesicles. [Supported by NIH grant GM-34399 and American Heart Association Grant-in-Aid 88-0721.]

88. Comparison of Transient Behavior of Native vs. Nystatin-induced Apical Channels in Isolated Frog Skin

T. HOSHIKO and STEFAN MACHLUP,* *Department of Physiology and Biophysics and Department of Physics, Case Western Reserve University, Cleveland, Ohio*

A paradox about the rate of closing of sodium channels remains unresolved. On the one hand, the current peaks after jumps in apical [Na] decay with time constants of a few seconds. On the other hand, the spectrum of sodium noise shows no corner frequency even well below 0.1 Hz. A noiseless diffusion transient would resolve the paradox. Evidence in favor of a diffusional/compart-

mental mechanism is found in the similarities in kinetic behavior between the nystatin-induced channels in the apical surface and the native sodium-selective channels. Nystatin channels exhibit a large overshoot in response to an apical [Na] or [K] concentration jump, very similar to that exhibited by the apical surface of untreated frog skin. Both the nystatin-induced and native channels exhibit a current undershoot when the apical [Na] is washed out. Another piece of evidence consistent with a compartmental theory is as follows. When preequilibrated for increasing periods of time in low [Na], the subsequent peak overshoot currents increase, reaching a maximum at a preequilibration period in low [Na] of ~120 s.

89. Aberrant Regulation of the Hexose Transport System in Retinoblastoma Y-79 DONNA ULLREY* and HERMAN M. KALCKAR, *Department of Chemistry and Biochemistry, Boston University, Boston, Massachusetts*

Human retinoblastoma Y-79 has been found to be insensitive to certain growth factors and unlike certain related tumors such as neuroblastomas, Y-79 lacks TGF-β receptors (Kimchi et al. 1988. *Science.* 240:198). We have studied a type of down-regulation of the hexose transport system that is prevailing in a large variety of mammalian fibroblast cultures, including human fibroblast cultures. This type of down-regulation, which is exerted by glucose and D-allose, but not by fructose, we have named the glucose-or allose-mediated "curb" (see review by Kalckar and Ullrey, editor. 1986. *In* Carbohydrate Metabolism in Cultured Cells. M. J. Morgan, Plenum Press). This type of curb has also been found in rat myoblast cultures (see review). Neither glucose nor allose manifest any trace of a hexose transport curb in Y-79 based on hexose-free or fructose-containing media. In relative terms basing the hexose transport activity on fructose media as 1.0, we found that the activity in glucose media was 0.9, and in allose media 0.9 as well. This is in contrast to corresponding quotients on the hexose transport activity which we have found in fibroblast cultures, tumorigenic or nontumorigenic, in which the corresponding scores using fructose-fed cultures as 1.0, were glucose-fed cultures: 0.5–0.3, allose-fed cultures: 0.5–0.2 (Ullrey and Kalckar. 1987. *Proc. Natl. Acad. Sci.* 84:3678). The maintenance of attached cultures of Y-79 over a week or two, is more dependent on glucose or fructose than on the presence of L-glutamine. The cultures form large amounts of lactic acid from glucose, whereas fructose seems a more economical carbohydrate source. (Ullrey, unpublished observations). [Supported by National Eye Institute grant 1RO3EY07652-01]

90. Monitoring Mitochondrial and Plasma Membrane Potentials in Cultured Cells with a New ^{99m}Tc-based Lipophilic Cation MARY L. CHIU,* JAMES F. KRONAUGE,* and DAVID PIWNICA-WORMS, *Department of Radiology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts*

Hexakis (methoxyisobutylisonitrile) technetium(I) ([^{99m}Tc]MIBI$^+$) is representative of a new class of myocardial perfusion imaging agents used for the noninvasive evaluation of coronary artery disease in patients. Chemical analysis of these complexes with the ground state ^{99}Tc isotope shows them to be monovalent cations with a central Tc(I) core octahedrally surrounded by six identical lipophilic ligands coordinated through the isonitrile carbon. Thus, these compounds are lipophilic cations, which raises the possibility that their mechanism of cellular uptake may involve distribution across biological membranes in response to membrane potential. To test this hypothesis, MIBI$^+$ uptake and retention were determined in cultured mouse BALB/c 3T3, NIH 3T3, and v-src transformed NIH 3T3 fibroblasts as well as in cultured chick embryo heart cells. Depolarizing plasma membrane potential (E_m) with 130 mM K, 20 mM Cl buffer decreased MIBI$^+$ cell uptake in all preparations; resting E_m calculated from normal K/high K uptake ratios and the Nernst equation were -16 ± 3 mV and -14 ± 3 mV in NIH 3T3 and transformed NIH 3T3 fibroblasts, respectively, and time-averaged E_m was -42 ± 3 mV in spontaneously contractile heart cells. Addition of the potassium ionophore valinomycin (1 μg/ml) to NIH 3T3 cells depolarized in high K buffer further depressed MIBI$^+$ uptake, demonstrating the contribution from the mitochondrial compart-

ment alone. The protonophore carbonylcyanide-*m*-chlorophenylhydrazone (CCCP; 5 μM) caused the rapid release of cellular $MIBI^+$ from BALB/c 3T3 cells to the levels of nonspecific binding found in freeze-thawed preparations. Hyperpolarizing mitochondrial and plasma membrane potentials in BALB/C 3T3 cells with the K^+/H^+ exchanger, nigericin (5 μg/ml), increased $MIBI^+$ uptake. BALB/C 3T3 cells hyperpolarized in the presence of nigericin plus ouabain showed increased retention of $MIBI^+$ during washout experiments. These results indicated that cellular uptake and retention of $MIBI^+$ in these preparations were determined by both mitochondrial and plasma membrane potentials in a manner analogous to that of other well known lipophilic cations such as tetraphenylphosphonium. The gamma-emitting properties of [^{99m}Tc]$MIBI^+$ further raises the possibility of monitoring membrane potential in vivo. [Supported by a grant from the Whitaker Health Science Fund. D.P.-W. is a Radiological Society of North America Scholar.]

91. Glucose Transport in Skeletal Muscle Plasma Membrane Vesicles from Control and Exercised Rats PATRICIA KING, MICHAEL HIRSHMAN,* ELIZABETH HORTON,* and EDWARD HORTON,* *Department of Medicine, University of Vermont, Burlington, Vermont*

Glucose uptake by muscle tissue is dramatically stimulated by exercise. The enhanced transport may result from an increased number of transporters in the plasma membrane and/or changes in transporter intrinsic activity. In the present study, we measured the initial rates of transport for carrier-mediated D-glucose influx in skeletal muscle plasma membrane vesicles prepared from control (CPM) and immediately postexercised (EPM) rats (1 h of treadmill running) and compared these data to transporter number measured by cytochalasin B binding. Influx of [^{14}C]-D-glucose and [^{3}H]-L-glucose was assayed at 25°C under equilibrium exchange conditions over a range of glucose concentrations (1–60 mM in HEPES-buffered Ringers, pH 7.6) using a rapid filtration technique. Uptake was measured at four time points over 5 s and carrier-mediated transport rates were calculated as the difference between the initial rates of D- and L-glucose influx. The carrier-mediated glucose influx was inhibited by cytochalasin B, phloretin, and preincubation of the vesicles with trypsin. In addition, facilitated transport was cation independent (Na vs. K) and displayed transstimulation. The mean vesicular volumes (uptake at equilibrium) were not different for CPM and EPM and showed an osmotic dependence. The V_{max} for transport was 3.7-fold greater in vesicles from the exercised animals, 3.5 ± 0.4 vs. 13.0 ± 1.4 nmol/(mg protein × s) for CPM and PEM (*mean* ± SE). $K_{1/2}$ values were not different, 20 ± 4.6 mM (CPM) and 22 ± 5.5 mM (EPM). Transporter number was increased 1.7-fold by exercise from 5.4 ± 0.7 to 9.4 ± 0.4 pmol/mg protein (CPM vs. EPM). As a result, the carrier turnover number increased 1.9-fold, 719 ± 15 s^{-1} (CPM) vs. 1,380 ± 158 s^{-1} (EPM). These data provide evidence that the response of glucose transport to exercise involves an increase in the average carrier intrinsic activity as well as a recruitment of transporters to the plasma membrane. [Supported by NIH grant DK-26317.]

92. Systemic Hydroxyurea Treatment Increases Red Cell Water Content in Dogs: Implications for Therapy of Sickle Cell Disease JOHN C. PARKER and EUGENE P. ORRINGER,* *Department of Medicine, The University of North Carolina at Chapel Hill, North Carolina*

Hydroxyurea, a drug useful in cancer chemotherapy, promotes the synthesis of hemoglobin F. Since hemoglobin F is a known inhibitor of hemoglobin S polymerization, hydroxyurea has recently been given to patients with sickle cell disease. Preliminary results show a reduction in pain crises and an improvement in anemia (Charache et al. 1987. *Blood.* 69:109). A second, well-known effect of hydroxyurea is a gradual increase in mean red cell volume (MCV). If the rise in MCV represented an increase in red cell water, then an additional explanation for the therapeutic effect of hydroxyurea in sickle cell disease might involve the well-known relationship between cell hydration and the kinetics of sickling: the more water there is in the red cell, the lower the cytoplasmic concentration of hemoglobin S, and the longer the delay time between deoxygenation and gel formation (Hofrichter et al. 1974. *Proc. Natl. Acad. Sci.* 71:4864). We gave hydroxyurea by mouth to two

dogs at doses of 25 and 50 mg/kg per d and made serial measurements of MCV, mean cell hemoglobin concentration (MCHC), cell ion and water content, and cell density using a phthalate technique. In the dog receiving the higher dose, red cells changed over the first 54 d as follows: MCV increased from 72 to 89 fl, MCHC decreased from 35 to 30 g/dl, cell water rose from 65.9 to 67.6% wet weight, and mean cell density fell from 1.103 to 1.098. Cell Na and K concentrations (mmol/kg wet cells) did not change, but Na content (mmol/kg dry weight) increased from 257 to 269. The dog receiving the lower dose had more modest changes in the same direction: MCV from 72 to 78 fl, MCHC from 34 to 32 g/dl, and cell water from 65.4 to 66.6% wet weight. In neither dog was there an effect of hydroxyurea on the reticulocyte percentage or on the white cell or platelet counts. Both animals maintained hemoglobin values of 15 g/dl or better, white blood counts of at least 5,000 per μl, and platelet counts in excess of 200,000 per μl. We conclude that systemic hydroxyurea therapy causes a gradual increase in red cell ion and water content, perhaps by altering the set point of transport mechanisms responsible for volume regulation. It is unlikely that the effect of the drug on dog red cells is mediated through potassium transport pathways since these cells have no Na-K pump and remain low in their content of potassium during treatment. [Supported by USPHS grants 5 RO1 DK11356 and 2 P60 HL28391.]

93. Regulation of Contact Sites between Rhodopsin and Transducin by Guanine Nucleotides HEIDI E. HAMM,* *Department of Physiology and Biophysics, University of Illinois College of Medicine, Chicago, Illinois* (Sponsor: Peter Reiser)

The structural basis for the high-affinity binding of transducin (G_t) to photoexcited rhodopsin (R*) has been recently reported (Hamm et al. 1988. *Science.* 241:832). Synthetic peptides corresponding to two regions of the G_t α subunit, α_t-311-328 and α_t-340-350 at the carboxyl terminal are competitive inhibitors of R-G_t interaction by virtue of their ability to bind rhodopsin and occupy G_t-binding sites on the surface of rhodopsin. These studies suggest that the carboxyl terminal of α_t interacts directly with rhodopsin in the high-affinity R*-$G_t\alpha$(empty pocket)-$\beta\tau$ conformation. GTP and GDP are equipotent in inducing a low-affinity state of hormone binding in a variety of tissues. We are interested in determining whether GDP also induces low affinity between R*- and G_t, and whether similar or different regions on the α_t surface are involved in the R*-$G_t\alpha$(GDP)-$\beta\tau$ conformation. The accessibility of the carboxyl terminal of G_t to MAb 4A and pertussis toxin, which both have binding sites in this region, was determined in the absence and presence of GDP, GDPβS, and GTPτS. In the absence of guanine nucleotides, the α_t carboxyl terminal was hidden; MAb 4A did not bind and PT did not ADP-ribosylate G_t. In the presence of GDP or GDPβS, the ability of MAb 4A to bind and PT to ADP-ribosylate G_t were restored. However, the affinity of interaction between R* and G_t-α(GDP)-$\beta\tau$ was as high as R*-$G_t\alpha$ (empty pockets) $\beta\tau$, assessed using binding to membranes in low ionic strength washes. This suggests that regions other than the α_t carboxyl terminal are involved in this interaction. [Supported by NIH EY-06062 and NSF DMB-8804861.]

94. Proteins and Regulation of Secretion: Amylase Release in Pancreatic Cell Line AR4 Stimulated by Substance P and Angiotensin II WING-TAI CHEUNG* and MICHAEL R. HANLEY,* *Molecular Neurobiology Unit, University of Cambridge Medical School, Cambridge, England*

Amylase release in the rat pancreatic acinar cell line, AR4-2J, is a model of physiological secretion pathways coupled to peptide receptors. Substance P (SP) and angiotensin II (AII) are potent secretagogues in this cell line (ED_{50} for SP = 1 pM, ED_{50} for AII = 300 pM), which work through a common transduction pathway, the breakdown of inositol phospholipids. Several lines of evidence suggest, however, that SP and AII receptors may be coupled to phospholipase C by distinct G proteins. High affinity GTPase can be activated in membranes by SP or AII, but AII exhibits a bell-shape dose-response curve. Neither SP- nor AII-stimulated amylase release is altered by cell pretreatment with either cholera toxin or pertussis toxin. However, SP and AII appear to differ in their influence over basal and toxin-catalyzed ADP-ribosylation of substrate proteins. These results imply an unexpected heterogeneity in the pertussis-insensitive G protein which regulates phospholipase C activity.

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